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Systemic and Colonic Hemodynamic and Vasomotor Responses to Adenosine Triphosphate in Horses.

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SYSTEMIC AND COLONIC HEMODYNAMIC AND VASOMOTOR RESPONSES TO ADENOSINE TRIPHOSPHATE IN HORSES

A Dissertation

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To all of the individuals and

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A	artery/arterial
AA	antimycin A
AA/D	antimycin A depleted
AA/R	antimycin A repleted
ADP	adenosine diphosphate
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMP	adenosine monophosphate
APC	atrial premature contraction
AST	aspartate transaminase
ATP	adenosine triphosphate
ATP-$MgCl_2$	adenosine triphosphate and magnesium chloride
AUC	area under the curve
AV	atrioventricular
BL	baseline
BUN	blood urea nitrogen
C	control
°C	centrigrade
$CaCl_2$	calcium chloride
CaO_2	systemic arterial oxygen content
CI	cardiac index
CK	creatine kinase
CMP	colonic mucosal perfusion
cNOS	constitutive form of nitric oxide synthase
CNS	central nervous system
CO	cardiac output
CO_2	carbon dioxide
cpu	capillary perfusion units
CSP	colonic seromuscular perfusion
CV	coefficient of variation
CVP	central venous pressure
DAG	diacylglycerol
DAP	diastolic systemic arterial pressure
DBFP	doppler ultrasound blood flow probe
DC	dorsal colon
DCAC	dorsal colon arterial catheter
DCAP	dorsal colonic arterial pressure
DCF	dorsal colonic blood flow
DCR_L	dorsal colonic vascular resistance
DCVC	dorsal colon venous catheter
DCVP	dorsal colonic venous pressure
DO_2	oxygen delivery
DPAP	diastolic pulmonary arterial pressure
ECG	electrocardiogram

EDHF	endothelium-derived hyperpolarizing factor
EJP	excitatory junction potential
endo +	endothelium intact
endo -	endothelium denuded
eNOS	endothelial form of nitric oxide synthase
ET-1	endothelin-1
G_i	inhibitory G protein
GGT	γ-glutamyl transferase
Hb	hemoglobin
$HCLO_4$	perchloric acid
HCO_3^-	bicarbonate ion
HPLC	high performance liquid chromatography
HR	heart rate
IL	interleukin
IP_3	inositol 1,4,5-triphosphate
IV	intravenous
KCl	potassium chloride
L/A	LPS/ATP-$MgCl_2$
LC	liquid chromatography
LDMFP	laser doppler mucosal flow probe
LDSFP	laser doppler serosal flow probe
LN	endo + vessel incubated with 10^{-4} M L-NAME
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
L/S	LPS/0.9% NaCl
MAP	mean systemic arterial pressure
MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium chloride
MPAP	mean pulmonary arterial pressure
MRAP	mean right atrial pressure
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	sodium phosphate monobasic
Na_2HPO_4	sodium phosphate dibasic
NANC	non-adrenergic non-cholinergic
NaOH	sodium hydroxide
NH_3	ammonia
NO	nitric oxide
NOS	nitric oxide synthase
NT	no tissue
O_2	oxygen
OCAP	overall mean colonic arterial pressure
OCR_L	overall mean colonic vascular resistance
OCF	overall mean colonic arterial blood flow
OCVP	overall mean colonic venous pressure

OD	outside diameter
P_i	orthophosphate
$PaCO_2$	partial pressure of carbon dioxide
PaO_2	partial pressure of oxygen
PAP	pulmonary artery pressure
PCV	packed cell volume
PF	pelvic flexure
PGI_2	prostacyclin
PI	post-infusion
PK	protein kinase
PL	phospholipase
PP_i	pyrophosphate
PR_L	pulmonary vascular resistance
PVC	premature ventricular contraction
PWP	pulmonary wedge pressure
RBC	red blood cell
RT	retention time
RDC	right dorsal colon
RVC	right ventral colon
S/A	0.9% NaCl/ATP-$MgCl_2$
Sao_2	percentage oxygen saturation
SAP	systolic systemic arterial pressure
SC	subcutaneous
SCRS	substrate-containing Ringer's solution
SEM	standard error of the mean
SFRS	substrate-free Ringer's solution
SPAP	systolic pulmonary arterial pressure
SR_L	systemic vascular resistance
S/S	0.9% NaCl/0.9% NaCl
SV	stroke volume
TCO_2	total carbon dioxide
TGF-β	transforming growth factor-β
TJ	tight junction
TM	transmembrane
TNF	tumor necrosis factor
V	vein/venous
VC	ventral colon
VCAC	ventral colon arterial catheter
VCAP	ventral colonic arterial pressure
VCF	ventral colonic blood flow
VCR_L	ventral colonic vascular resistance
VCVC	ventral colon venous catheter
VCVP	ventral colonic venous pressure
VFA	volatile fatty acid
WBC	white blood cell

ABSTRACT

Clinically healthy horses were instrumented to measure hemodynamic, metabolic, hematologic, and serum biochemical variables and monitor clinical signs during and after IV infusion of ATP-$MgCl_2$. Conscious horses were administered ATP-$MgCl_2$ (0.05 mg ATP/kg body weight/min; 0.05 mg/kg/min increments; maximum rate - 1.0 mg/kg/min), which caused a rate-dependent increase in cardiac output, decrease in systemic vascular resistance, and mild pulmonary hypertension. The maximal safe infusion rate was 0.3 mg ATP/kg body weight/min. Anesthetized horses administered ATP-$MgCl_2$ (0.1 to 1 mg/kg/min; 0.1 mg/kg/min increments) developed a rate-dependent decrease in systemic and colonic vascular resistance via vasodilatation. In conscious horses administered low-dose endotoxin, ATP-$MgCl_2$ (dose - 100 μmole/kg ATP and 100 μmole/kg $MgCl_2$; rate - 0.3 mg/kg/min) failed to attenuate the clinical, hemodynamic, metabolic, and hematologic alterations that occur secondary to endotoxin exposure; ATP-$MgCl_2$ infusion appeared to potentiate pulmonary hypertension, leukopenia, and neutropenia observed with endotoxin.

Based on the results of the in vivo studies, the effects of ATP on vasomotor tone of isolated equine colonic arterial and venous rings were studied. Non-cumulative dose response curves of vessel rings from normal horses to ATP (10^{-8} to 10^{-3} M) were generated in the presence and absence of endothelium and in the presence of a non-specific nitric oxide synthase inhibitor, L-NAME (10^{-4} M). ATP caused a biphasic response at high doses (10^{-4} and 10^{-3} M) in both vessel types, an initial transient contraction followed by a slow, substantial and sustained relaxation, which was

attenuated with endothelium removal. The endothelium-dependent relaxation component was mediated by a mechanism other than nitric oxide.

A method to quantify adenine nucleotides in equine colonic mucosa was validated. Adenine nucleotides were stable in lyophilized tissue stored at -70 C for at least 54 days. Effects of an electron transport inhibitor (antimycin A) on mucosal nucleotides was determined in a whole tissue model. In the presence of glucose and oxygen, ATP was stable for up to 4 hrs, but ADP and AMP decreased. In the absence of glucose, ATP remained stable for only 3 hrs. Antimycin A (50 μM) caused a time-dependent, irreversible decrease in adenine nucleotides.

CHAPTER 1. INTRODUCTION/REVIEW OF LITERATURE

1.1 Introduction

Acute gastrointestinal tract disease is the leading natural cause of death in adult horses. Strangulating volvulus of the ascending colon is a common cause of gastrointestinal tract ischemia and is associated with substantial morbidity and mortality. Loss of fluid into the peritoneal cavity and intestinal lumen leads to hypovolemia. Additionally, splanchnic ischemia causes mucosal barrier disruption and transmural migration of bacteria and endotoxin into the systemic circulation. The combination of endotoxemia and hypovolemia causes profound alterations in the circulatory system with the development of circulatory shock. If left untreated, affected animals will progress to multiple organ failure and death.

Adenosine triphosphate (ATP) is the principal immediate donor of free energy for mammalian cells. Additionally, ATP and its catabolites, have profound effects on vasomotor tone via interaction with specific purinergic receptors. During tissue ischemia and shock, endogenous production of ATP is decreased, which can profoundly alter the microcirculation, cell membrane transport and function and cellular energy metabolism.

Administration of a combination of ATP and magnesium chloride (ATP-$MgCl_2$) has been demonstrated to alter vasomotor tone by producing vasodilatation, thereby potentially enhancing blood flow, microcirculation and tissue perfusion. Additionally, ATP-$MgCl_2$ has been shown to improve tissue ATP content, organ function, and down-regulate the synthesis of cytokines and inflammatory mediators. However, the efficacy of ATP-$MgCl_2$ during gastrointestinal tract disease and endotoxemia in horses is not known.

1.2 Circulatory Shock

Despite many advances in cardiovascular support therapy, circulatory shock remains an important cause of death in both humans and domestic animals. Circulatory shock can be defined as "a general inadequacy of blood flow to tissues relative to their metabolic demands" (1). If circulatory shock is inadequately managed, the result is multiple organ failure, particularly the lung and splanchnic organs, and ultimately death (1).

Two main classification systems have been proposed to categorize circulatory shock. The first classification system ascribes the pathologic events to the initiating insult, such as hemorrhage, trauma, sepsis, and myocardial infarction (1). The second classification system groups the insult according to the character of the predominant circulatory disturbance: cardiogenic, obstructive, hypovolemic, and distributive (2,3).

The most common forms of shock observed in the horse are hypovolemic and distributive (4). Hypovolemic shock refers to a loss of intravascular volume associated with the loss of whole blood, plasma, or largely protein-free fluid through sweat, diarrhea, or urine (1). The principal alterations that occur during non-hemorrhagic hypovolemic shock include arterial vasoconstriction, decreased venous pressure, hemoconcentration, tachycardia and oliguria (5). Because of the decrease in vascular filling volume, venous return to the heart decreases, with a subsequent decrease in cardiac output (CO), arterial flow, and arterial pressure (1).

Distributive shock refers to an expansion of the vascular space owing to regional or generalized loss of vascular tone (1). Principal causes of distributive shock include sepsis, neurologic disturbance, anaphylaxis or metabolic, toxic or endocrinologic

depression of vasomotor tone (5). A common feature of distributive shock is inappropriate vasodilatation, which leads to maldistribution of vascular volume and impaired organ perfusion (5). In some forms of distributive shock, such as during the early stages of septic shock, CO and total organ blood flows may be increased, but at the microvascular level, there may be failure to match blood flow to local parenchymal demands, which leads to tissue hypoxia and dysfunction (1).

From a clinical perspective, many diseases fit into more than one classification system. For example, gastrointestinal tract ischemia associated with equine colic leads to hypovolemic shock owing to loss of peritoneal and intestinal luminal fluid (1). When gastrointestinal mucosal barrier disruption occurs secondary to ischemia, transmural migration of bacteria and endotoxin into the portal and systemic circulations will lead to distributive shock (1). In adult horses, the most common cause of distributive shock is endotoxemia, which principally occurs secondary to splanchnic ischemia associated with acute strangulating and nonstrangulating intestinal disease (1).

When endotoxin enters the portal and systemic circulations, it can induce an inflammatory response via both direct and indirect mechanisms. Endotoxin can directly activate the complement, coagulation and fibrinolytic cascades (6-10). Indirect mechanisms include activation of numerous cell types, such as lymphocytes, neutrophils, endothelial cells, platelets and mononuclear cells, which perpetuate the inflammatory response (11).

When mononuclear cells are activated by endotoxin, they liberate cytokines, principally tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6 (12). Cytokines are small (8-30 kD), intercellular messenger polypeptides that are active at low

concentrations (12). Cytokines possess growth-promoting, proinflammatory or anti-inflammatory effects. The primary proinflammatory cytokines include IL-1, IL-8 and TNF (12). When produced in excess, the primary cytokines initiating adverse effects are IL-1 and TNF (13-17). The principal effects of these cytokines are diffuse microvascular plugging secondary to activation of the coagulation system (9), loss of vascular autoregulation, and increased vascular permeability (18).

The primary anti-inflammatory cytokines are IL-10, IL-6, IL-1ra and transforming growth factor (TGF)-β (12). In the course of overwhelming inflammation, these natural anti-cytokine mediators are produced in insufficient quantities to be capable of effectively containing the insult (12).

Tumor necrosis factor is referred to as "THE shock cytokine" (12). There are 3 members of the TNF gene family: TNF-α, TNF-β, and lymphotoxin-β (19-23). The pathogenic role of TNF-α is well-documented (24). It is a 17 kD polypeptide released principally by activated macrophages in response to lipopolysaccharide (LPS) (14,25). This cytokine increases rapidly and peaks at 60-90 minutes after endotoxin exposure (12). The principal activities of TNF-α include induction of other cytokines such as IL-1, IL-4 and IL-6; activation of T cells; endogenous pyrogen activity, induction of endothelial cell surface antigens and procoagulation activity; eicosanoid synthesis; activation of osteoclastic bone resorption; inhibition of bone collagen synthesis; induction of acute-phase reactant synthesis and granulocyte/monocyte stimulating factor; and inhibition of enzymes involved in lipid metabolism (26-28). In neutrophils, TNF-α stimulates activation of the respiratory burst, degranulation and adherence to

vascular endothelium (29). Additionally, TNF-α can cause myocardial depression, hypercoagulability, hypotension and death (12).

Interleukin-6 is a phosphoglycoprotein molecule synthesized by numerous cell types in response to various stimuli, including endotoxin, IL-1, and TNF-α (30). It peaks at 120-180 minutes following exposure to endotoxin and possesses both anti- and proinflammatory activities (12). It functions as a suppressor of proinflammatory cytokine production, a pyrogen, and an initiator of the acute-phase response (12). Since IL-6 circulates in high concentrations, it is the best cytokine marker of inflammatory disease activity (12).

Interleukin-1 is generated primarily by mononuclear phagocytes (31). It possesses many of the same biological activities of TNF-α, peaks at 180 minutes after exposure to endotoxin, and can stimulate the synthesis and release production of TNF-α, IL-8, and IL-6 (12,32).

Secretion of cytokines, most notably IL-1 and TNF-α, affect the temperature set-point, vascular resistance and permeability, cardiac function, bone marrow and enzyme systems (12). Many effects of cytokines are mediated at the target tissues by other inflammatory mediators, such as nitric oxide (N0) and eicosanoids (12). IL-1 and TNF-α stimulate elaboration of other cytokines, which then amplify and modulate the cytokine response (33,34).

Other inflammatory mediators produced during endotoxic shock include arachidonic acid metabolites (prostaglandins, thromboxanes, and leukotrienes), platelet activating factor, NO, endothelin-1 (ET-1), proteases, and reactive oxygen species

(11,12,35-39). These various mediators can profoundly affect vasomotor tone, coagulation, and leukocyte-endothelial interactions.

When neutrophils are exposed to endotoxin and other inflammatory mediators, they undergo functional alterations and release proteolytic enzymes (40) and reactive oxygen radicals (41), which cause local endothelial and tissue injury. Endotoxin and TNF stimulate neutrophil migration (42) and endothelial adhesion via up-regulation of expression of adhesion molecules on the cell surface of both neutrophils and endothelial cells (42-44). Additionally, neutrophil deformability decreases, which favors additional sequestration in the microvasculature (45,46).

Vascular endothelium is one of the target organs that is adversely affected during endotoxic shock (47,48). Release of tissue thromboplastin and exposure of subendothelial collagen that occurs secondary to endothelial cell damage activates both the extrinsic and intrinsic coagulation cascades (31). Endotoxin causes both structural and metabolic changes in endothelial cells and increases permeability of the endothelial cell layer (49). Additionally, release of endothelium-derived mediators, such as NO, ET-1, and prostaglandins, contribute to the inflammatory and hemodynamic derangements that occur during endotoxic shock.

During the initial stages of shock (hyperdynamic phase), myocardial contractility and heart rate increase and peripheral vasoconstriction occurs (5). These hemodynamic alterations occur secondary to sympathetic activation in an attempt to maintain blood pressure and increase vital organ perfusion by redistributing flow away from skin, skeletal muscle, and renal and splanchnic beds (5). Activation of the renin-angiotensin system leads to additional vasoconstriction and increases in vascular volume due to

sodium retention (5). Numerous other mediators are released in an attempt to maintain an energy source for cells and intravascular volume to maintain blood flow (5).

An early consequence of decreased oxygen availability during shock is a reduction in cellular ATP content (5). When tissue oxygen tension decreases to a critical level, oxidative phosphorylation is uncoupled and ATP production gradually ceases (50). Depletion of ATP ultimately leads to mitochondrial swelling, cell membrane deterioration, abnormalities in cellular calcium flux, intracellular accumulation of Na^+, Ca^{++}, and water, and lactic acidosis (5). In an attempt to offset the changes that occur with decreased ATP content, administration of ATP-$MgCl_2$ has been shown to attenuate some of the adverse metabolic alterations that occur during shock (50) (Refer to section on ATP-$MgCl_2$ for additional information).

Among adult horses, particularly if shock is associated with splanchnic ischemia, many of the early hyperdynamic events are not observed (1). During the later stages of shock (hypodynamic phase), the alterations that occur in horses are similiar to humans and other species. Hypovolemia develops secondary to arterial and venous dilatation and leakage of plasma into the extravascular space (51,52). Initially, despite a normal or increased CO, ventricular function is abnormal leading to reduction in the ejection fraction (52,53). The hypovolemia and vasodilatation decreases systemic vascular resistance (SR_t), and there is decreased peripheral use of oxygen and other nutrients by the cells (54-56). Perfusion may be normal or decreased but cellular metabolism is decreased (52). Maldistribution of blood flow occurs due to constriction of some vascular beds and vasodilatation of others (52). Enhanced leukocyte adherence (53,57,58) and neutrophil and platelet aggregation can lead to a further decrease in

blood flow owing to microvascular plugging (59). Additionally, increased intravascular coagulation occurs (56-58). Eventually multiple organ failure develops (52,56). If the hemodynamic derangements are not reversed, death ultimately ensues.

In horses, injection of lethal (50-200 μg/kg) and sublethal (10-40 μg/kg) doses of endotoxin causes a decrease in systemic arterial pressure (SAP) and CO and an increase in right atrial pressure, pulmonary artery pressure (PAP) and pulmonary vascular resistance (PR_L) (60-63). The decreased CO output likely is associated with depressed cardiac mechanical function, decreased venous return, increased PR_L or a combination (64-66). Splanchnic vasoconstriction occurs as part of the compensatory response to endotoxemia (66,67). Intestinal vasoconstriction has been documented in other species administered low-dose endotoxin (68).

The three main goals during the treatment of circulatory shock are: restoration of organ perfusion; control or reversal of the initiating cause; and prevention or management of the complications associated with shock (5). The first and most important therapeutic goal is restoration of blood volume (69). The initial aim is to restore organ perfusion in order to provide adequate oxygen delivery to the tissues to meet their metabolic needs (5). Restoration of arterial blood pressure, vascular volume and CO is of paramount importance in supporting tissue oxygenation (5). Frequently, patients have maldistributed blood flow with decreased intravascular volume but with increased interstitial water (70-73). Therapy should be aimed at improving circulatory function by restoring plasma volume, not by overloading an already expanding interstitial space (69).

Blood volume expansion is the most important element of therapy for circulatory shock (5). The easiest and most effective method to achieve intravascular volume expansion is with colloids, which expands plasma volume without overexpanding interstitial water (73-78). Plasma is the most effective colloid (5). Its effects are long-lasting and if fresh or fresh frozen plasma is used, it also provides clotting factors (5). If blood loss and hemodilution cause a significant decrease in oxygen-carrying capacity, whole blood is indicated (5). The use of dextrans (high molecular weight polysaccharides) or other synthetic colloid volume expanders, such as hydroxyethyl starch, are helpful in the absence of plasma (5).

Crystalloids can also provide blood volume expansion. However, when they are used, large fluid replacement volumes are required because only 25 to 30% of the crystalloid solution remains in the vascular space 30 minutes following administration (79). Because of the rapid diffusion from the vascular space, further fluid administration in the form of colloids may be needed if hypotension or vasoconstriction persists (5). Interstitial edema may develop, which can subsequently cause disturbances in organ function, especially the lung and splanchnic organs (80). The use of fluids high in sodium, such as 0.9% NaCl (sodium chloride), help maintain the fluid in the extracellular and intravascular space (5).

Measurement of central venous pressure (CVP) and pulmonary wedge pressure (PWP) are useful to determine the capacity of the vascular system to accept more volume without producing pulmonary edema and to prevent acute blood volume overload during rapid fluid restoration. Although CVP and PWP may accurately measure venous pressures, they do not accurately reflect blood volume in most patients.

The distribution of body water between plasma, interstitium and intracellular compartments can only be definitively measured using isotopic body composition studies (70-72).

In horses, the goal during volume replacement is to maintain a mean arterial pressure (MAP) of 80 mm Hg or greater and a CVP between 10-15 cm H_2O. A rapid increase in CVP may indicate too rapid hydration in the face of cardiac dysfunction. The use of hypertonic or hyperoncotic fluids during circulatory shock in horses will transiently shift fluid from the extravascular, extracellular space to the vascular space, thereby expanding plasma volume. The effect is temporary because the high concentrations of intravascular sodium and chloride ions rapidly traverse the vascular bed into the interstitium, returning fluid to the interstitial compartment. Therefore, administration of crystalloids or colloids along with hypertonic or hyperoncotic fluids is vital to keep the fluids in the intravascular space (1).

Under normal circumstances, the plasma acid-base status is maintained by plasma bicarbonate (HCO_3^-) and protein buffering systems, with acute respiratory and longer-term renal adjustments. Many cases of metabolic acidosis associated with circulatory shock will self-correct after adequate intravascular volume replacement has been effectively achieved (1).

The use of antimicrobial agents does not alter survival rate in endotoxic animals (81) but endotoxemia has been shown to cause translocation of bacteria from the gastrointestinal tract to other organs (82). Severe, protracted hypovolemic shock has been associated with impaired splanchnic perfusion with subsequent mucosal barrier

disruption and bacterial translocation (83). Therefore, the use of prophylactic broad-spectrum antimicrobial agents is advocated (1).

The use of inotropic and/or vasoactive drugs during circulatory shock may be warranted. If poor peripheral perfusion and low CO exists despite fluid therapy and CVP is increased, positive inotropic therapy should be initiated (5). Following volume expansion, the use of inotropic agents, such as dobutamine, may help achieve optimal cardiac index (CI), oxygen delivery (DO_2), and oxygen consumption (69). Dobutamine has been shown to cause a marked and significant increase in CI and stroke index, cardiac and stroke work, and DO_2 and consumption. Additionally, dobutamine decreases SR_L and PR_L, CVP, and PWP (78,84,85). Vasodilatory agents, such as acepromazine, may be indicated if MAP is normal or increased and if there is a high SR_L (69). If hypotension persists following volume expansion, the use of vasopressor agents may be warranted to maintain perfusion pressure (69).

The efficacy of corticosteroids to treat hypovolemic and septic shock has been extensively studied. Corticosteroids mediate multiple anti-inflammatory effects through alterations in gene transcription and translation (56). They significantly decrease the synthesis of TNF-α, which results in significant down-regulation of the inflammatory response (86). They increase the synthesis of lipocortin, a protein that inhibits phospholipase (PL) A_2 activity and the formation of arachidonic acid-derived eicosanoids (56). There are many potential disadvantages associated with the use of corticosteroids for the treatment of sepsis (56). Profound immunosuppression results from decreased neutrophil chemotaxis, antigen-antibody complexing, and opsonization, thus leading to refractory or even secondary infections (56). Corticosteroid

administration is reported to induce laminitis in some horses by potentiating catecholamine-induced digital vasoconstriction (87). This may particularly be important in patients that are already at risk of developing laminitis (56).

Early studies in experimental animal models demonstrated beneficial effects of corticosteroids during endotoxemia and septicemia (57). Clinical trials in humans did not show beneficial effects and sometimes showed adverse effects (88). Studies investigating the application of corticosteroids in equine endotoxemia have failed to show any therapeutic benefit (89,90). Additionally, most investigators agree that systemic corticosteroids are contraindicated as treatment for severe sepsis and septic shock in horses (1).

In horses with shock, the use of anti-inflammatory agents is indicated. Non-steroidal anti-inflammatory drugs have been shown to have a protective effect during endotoxic and splanchnic-ischemia shock via modulation of arachidonic acid pathway mediators (91,92). Inhibition of cyclooxygenase results in decreased synthesis of prostaglandins and thromboxanes, thereby improving vascular and coagulation function (56). Leukotrienes contribute to hemodynamic, hematologic and pulmonary manifestations during shock (56). The beneficial effects of lipoxygenase-inhibiting drugs during shock have been demonstrated in other species but not in horses (56).

Finally, prevention of multiple organ failure is critical. Supplemental oxygenation via nasal insufflation may be necessary if arterial oxygen tension falls below 85 mm Hg due to risk of hemoglobin desaturation, which leads to tissue hypoxia (1). Additionally, renal and hepatic support is vital to help maintain water balance and prevent accumulation of toxic metabolites (5). The maintenance of intravascular fluid

volume, glomerular filtration, and urine production is critical in the prevention of renal failure (93). Administration of intravenous (IV) fluids and maintenance of normal electrolytes is vital. If renal perfusion is diminished and the animal remains oliguric 10 to 12 hours after starting fluid therapy, administration of dopamine may improve renal blood flow and urine output (93). The use of loop diuretics, such as furosemide, may be indicated to promote diuresis (93). Additionally, blocking the $Na^+/K^+/2Cl^-$ co-transporter in the kidney with loop diuretics may protect the renal tubule cells by reducing their metabolic rate (93).

Management techniques for hepatic insufficiency are supportive. Fluid deficit and acid-base or electrolyte imbalances should be corrected by intravenous fluids (94). Administration of agents to reduce the production of toxic protein metabolites by enteric bacteria or interfering with their absorption may be indicated. Mineral oil, oral administration of poorly absorbable antibiotics, or lactulose can be used (95-98). Additionally, rations high in carbohydrates and low in protein are recommended (94,97).

1.3 Equine Ascending Colon

The anatomy, physiology and pathophysiology of the equine ascending colon is complex. During disease, significant alterations in anatomical characteristics and physiological functions occur. Knowledge of normal gross and microscopic anatomy, as well as physiology of digestion, absorption and secretion, provides the foundation for understanding the mechanisms involved in disease processes.

The equine ascending colon is 3 to 4.5 meters in length with a capacity of 55 to 130 liters (99,100). It forms a double horseshoe-shaped loop consisting of the right

ventral, left ventral, left dorsal and right dorsal components (101). The sternal and diaphragmatic flexures are located at the cranial aspect of the ventral and dorsal loops, respectively (101). The pelvic flexure (PF) joins the left ventral and left dorsal segments caudally (101). The diameter of the ascending colon varies depending on the segment, with the right dorsal component being the largest (50 cm) (101). The colon has longitiudinal muscle bands, teniae coli, that vary in number depending on the segment and these bands form sacculations called haustra coli (101).

The proximal (right ventral colon [RVC]) and terminal (right dorsal colon [RDC]) regions of the ascending colon are attached to the dorsal aspect of the abdominal cavity by the ascending mesocolon (101). The vascular supply to the dorsal colon arises from the right colic artery, which is a branch of the cranial mesenteric artery (99,100,102). The ventral colon is supplied by the colic branch artery, which arises from the ileocolic artery (99,100,102). Both arteries are located in the colonic mesentery and travel parallel to the colon to anastomose at the pelvic flexure (99,102). The arteries branch from the colonic vessels every 2 cm and anastomose with vessels lying orally or aborally to form a colonic rete before continuing into the colonic tissue (102). The function of the colonic rete is unknown, but may provide collateral blood supply (103). Vessels enter the submucosa through the tunica muscularis to form a submucosal arteriolar plexus (102). Arterioles ascend from the submucosal plexus to the mucosa, where an extensive capillary network forms around the colonic glands (102). The submucosal venous plexus and sparsely distributed venules drain the capillary network (102).

Histologically, the ascending colon has a thin mucosal layer with straight, shallow colonic glands separated by extensive lamina propria. Goblet cells are numerous. The outer muscularis externa is thickened into flat bands of smooth muscle and elastic fibers, the taenia coli. Elastic fibers predominate over muscle in the taenia coli. A thin, single-layered muscularis mucosa is present, and villi are absent. Scattered lymphoid nodules are evident. The cells lining colonic glands, in addition to goblet cells, are granular cells (101).

The equine ascending colon does not possess mucosal enzymes or an active transport mechanism for hexoses, amino acids or B vitamins (104). The primary function of the large intestine in adult horses is the production and absorption of volatile fatty acids (VFAs) via microbial digestion of soluble and insoluble carbohydrates (105). Up to 75% of energy requirements are met by products of microbial fermentation of carbohydrates in the cecum and ascending colon (106). Additionally, the large intestine secretes a volume of fluid equal to the extracellular fluid volume and recovers approximately 90 to 95% of it every 24 hours (107).

The normal colonic luminal microorganisms consist of ciliated protozoa, anaerobic bacteria and small numbers of Enterobacteriaceae sp. (108,109). The normal colonic pH is maintained at 6.0-6.8 and osmolarity of 300 mOsm/L (106,110,111). The end products of fermentation of soluble (starch) and insoluble (cellulose) carbohydrates are the VFAs, acetate, butyrate, and propionate, and carbon dioxide, methane and small amounts of lactate (112). Acetate is produced in the largest quantity but the ratio of acetate:propionate decreases as the ratio of soluble:insoluble carbohydrate increases (105). Periods of high VFA production are associated with a rapid increase in fluid

volume, production of large quantities of osmotically active organic acids and gas formation (105,107). Homeostatic mechanisms to buffer organic acids include transport of bicarbonate from the small intestine, secretion of bicarbonate from the large intestinal mucosa and rapid absorption of VFAs (112).

Approximately 97% of VFAs are dissociated (ionized) at the average pH of the equine large intestine (113). The ionized form is poorly absorbed compared with the lipid-soluble unionized form (113). In the colonic lumen, carbon dioxide combines with water to form carbonic acid, which dissociates into bicarbonate and hydrogen ions (114). The ionized VFAs combine with the hydrogen ions to form undissociated (unionized) VFAs, which are subsequently absorbed into the colonic epithelial cells (115). Within the epithelial cells, the VFAs can be metabolized or transported into the blood and carried to the liver for energy metabolism (115). As the VFAs are absorbed, bicarbonate accumulates in the intestinal lumen (111).

Undigested protein entering the large intestine is digested by microbial flora to ammonia (NH_3) (104). The NH_3 pools arise from microbial deamination of amino acids and from hydrolysis of non-protein nitrogen such as urea (104). The NH_3 together with a carbon skeleton can be used to synthesize microbial protein (104). The rate of microbial protein synthesis depends on readily available carbohydrate sources and serves as a major route of NH_3 disposal (104). The majority of protein degraded is lost in feces or the NH_3 is absorbed into the blood and utilized by the horse (converted to urea in the liver) (104,106).

The ascending colon undergoes periods of net fluid and electrolyte secretion alternating with periods of net absorption. Absorptive processes are confined to the

surface epithelium of the colon while the crypts are secretory. Large volumes of water and sodium are secreted. Net secretion occurs at a time when VFAs are rapidly produced and provide additional fluid and bicarbonate buffer to facilitate fermentation (104).

Gastrointestinal transport of fluid and electrolytes involve both passive and active mechanisms. Passive forces include the intrinsic permeability of the intestinal epithelial cells, the osmotic pressure gradient exerted by intestinal luminal contents, the electrical potential difference across the intestinal epithelial cells, the concentration gradient of solutes across the intestinal epithelial cells, and the pH of the luminal contents (116). Luminal pH affects the absorption of weak acids and bases because only nonionized acids or bases can passively diffuse across cell membranes (117).

The electrical potential gradient across the cell promotes the movement of an ion toward the side of the cell with the opposite electrical charge. In the equine colon, the potential gradient is 35 to 40 mV, with the serosal side being positively charged. In horses, the potential gradient favors passive chloride absorption but restricts passive sodium absorption (115).

Both primary and secondary mechanisms exist for active transport of ions (116). Primary active transport involves transport of an ion against its electrochemical gradient using energy derived from another cell membrane transport process or from glucose metabolism (114). The interior of the mucosal cell is approximately 30 mV negative with respect to the mucosal surface (114). This charge difference promotes sodium entry into the cell from the intestinal lumen (114). In the equine colon, sodium is proposed to be actively transported into the epithelial cell secondary to Na^+, K^+-ATPase

pump activity (114). The pump actively transports sodium out of the cell across the basolateral membrane and brings potassium into the cell (118). Additionally, sodium transport into the cell is enhanced in the presence of glucose (113). The energy for this transport is derived from the intracellular metabolism of glucose (111).

Secondary active transport systems utilize the free energy derived from passive diffusion of one ion down its electrochemical gradient to transport another ion against its electrochemical gradient (116). In horses, Na^+-H^+ and Cl^--HCO_3^- exchange systems have been identified (114).

Acute gastrointestinal tract disease (colic) is the leading natural cause of death in adult horses (119,120). Gastrointestinal tract ischemia commonly develops secondary to low-flow/no-flow conditions, with small intestinal volvulus or incarceration (121,122) and large colon volvulus (123-125) being common causes. In one study, strangulating obstructive lesions were associated with the highest mortality (75%) of all types of colic (126). Large colon abnormalities account for up to 50% of the horses that die or are euthanatized subsequent to colic (120-122).

In horses, strangulating volvulus of the ascending colon has been reported to have a mortality approaching 80% (125). The disease is characterized by colonic luminal obstruction and vascular occlusion secondary to the volvulus, thereby resulting in colonic ischemia, mucosal necrosis and vascular thrombosis (127). Colonic blood flow has been shown to remain significantly below baseline values for at least 4 hours after correction of complete arteriovenous occlusion in horses (128). The high mortality associated with colonic volvulus may be related to a sustained reduction of blood flow and hypoperfusion (due to increased vascular resistance) after surgical correction and

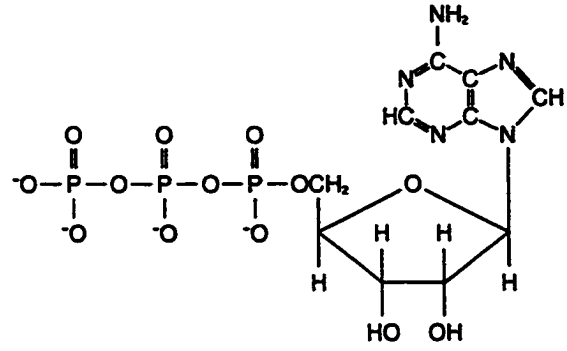
continued ischemic injury. Endothelial damage occurs in the colonic vasculature subsequent to ischemia-reperfusion and can be exacerbated by endotoxin (129). The sustained decrease in colonic blood flow may be associated with endothelial damage in the colonic circulation, leading to a loss of endothelium-derived vasorelaxants and subsequent vasoconstriction. Many of these horses develop systemic hypotension owing to hypovolemia and endotoxemia, which contribute to decreased splanchnic blood flow. Additionally, equine colonic mucosal ATP content has been shown to decrease 92% during ischemia and recovers to only 44% of control value after reperfusion, thereby limiting substrate availability for cellular metabolic functions (130). The decreased blood flow and tissue ATP content that occurs during colonic ischemia can lead to disruption of the mucosal barrier and transmural passage of endotoxin into the systemic circulation. If sufficient endotoxin enters the systemic circulation, death can ensue.

1.4 Biochemistry of ATP

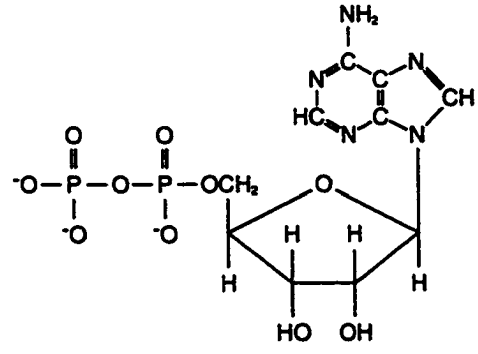
Mammalian cells require a continuous supply of energy to perform three basic functions: mechanical work, active transport of molecules and ions, and synthesis of biomolecules. Oxidation of foodstuffs provides free energy, which is subsequently transformed into a highly accessible and efficient energy form that is utilized by cells to perform the above-mentioned tasks. The free-energy donor in most energy-requiring processes is ATP. In 1941, Fritz Lipmann and Herman Kalckar first perceived the central role that ATP plays in energy exchange in biological systems (131).

ATP is an adenine nucleotide containing an adenine, a ribose, and a triphosphate unit (Figure 1.1). The active form of ATP is usually a complex of ATP with a co-factor,

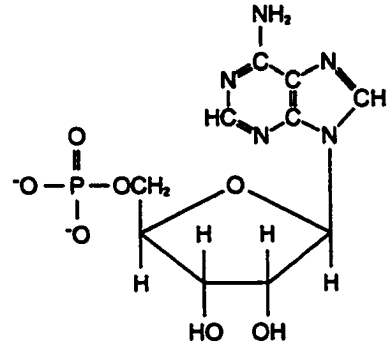
Figure 1.1 - Chemical structure of ATP, ADP, AMP and adenosine.



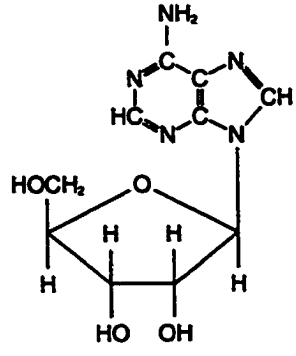
Adenosine triphosphate
(ATP)



Adenosine diphosphate
(ADP)



Adenosine monophosphate
(AMP)



Adenosine

either Mg^{2+} or Mn^{2+}. ATP contains two phosphoanhydride bonds in its triphosphate unit, which makes it a rich source of energy. When ATP is hydrolyzed to adenosine diphosphate (ADP) (Figure 1.1) and orthophosphate (P_i) or adenosine monophosphate (AMP) (Figure 1.1) and pyrophosphate (PP_i), large quantities of free energy are released (131).

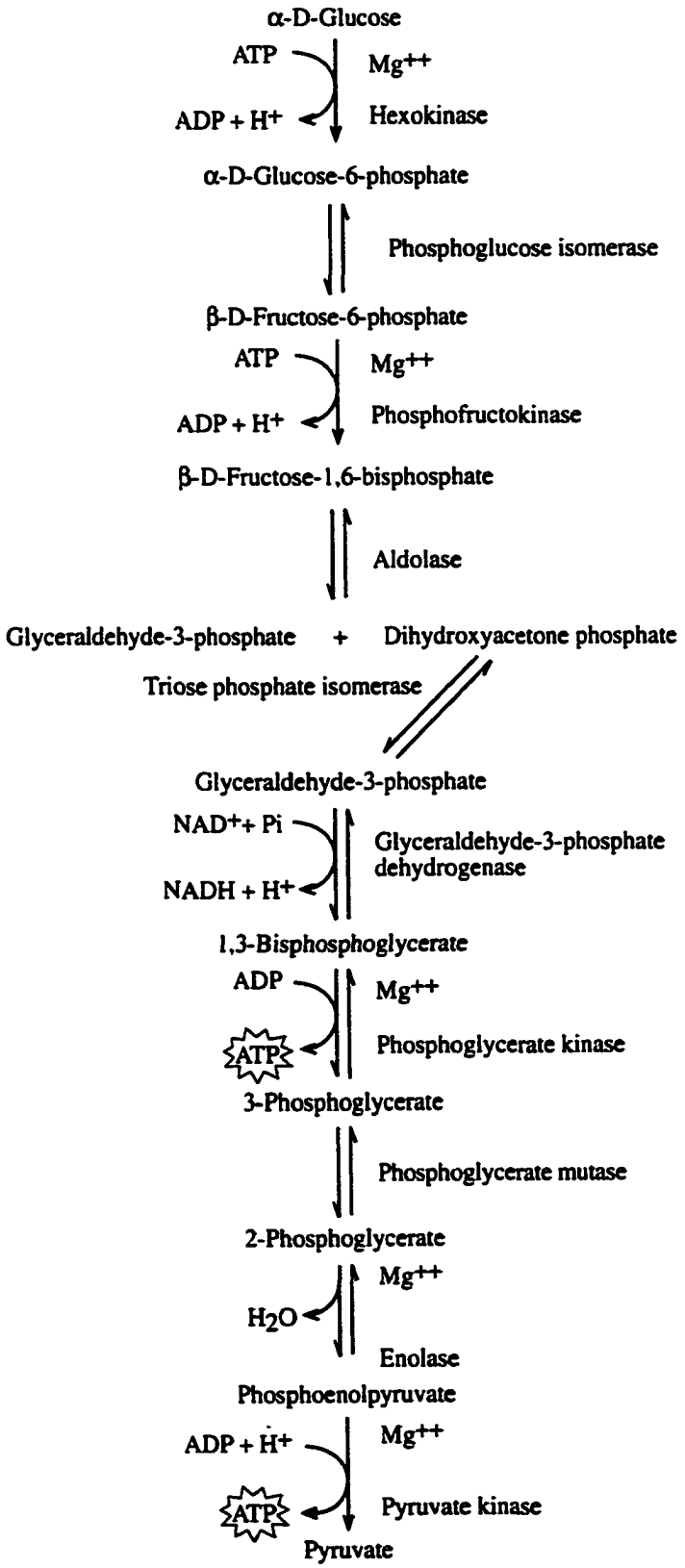
The adenine nucleotides are interconvertible. When ATP is hydrolyzed, the free energy liberated is used to drive reactions that require an input of free energy. When fuel molecules are oxidized, ATP is regenerated from ADP and P_i. This ATP-ADP cycle is the fundamental mode of energy exchange in biological systems (131).

In biological systems, ATP serves as the principal immediate donor of free energy rather than as a long-term storage form of energy. The turnover of ATP is immense. Motion, active transport, signal amplification and biosynthesis can occur only if ATP is continuously regenerated from ADP. Generation of a proton-motive force, which occurs when protons are pumped across a membrane, provides the power necessary to synthesize ATP. This is an extremely efficient, energy-conserving event that can generate large quantities of ATP (131).

Degradation of glucose to carbon dioxide and water is the principal source of ATP. Glycolysis is the initial step in which energy is released from the glucose molecule. The end products of glycolysis are then oxidized to provide energy. During glycolysis, one molecule of glucose is used to form two molecules of pyruvate (Figure 1.2). This process requires the input of two ATP molecules and a total of four molecules of ATP are formed. Therefore, the net ATP production per molecule of

Figure 1.2 - Glycolysis

Glycolysis



glucose utilized is two, with an overall efficiency of 43%. The remaining 57% of energy is lost in the form of heat (132).

Pyruvate formed from glycolysis is converted to acetyl-CoA, which enters the Citric Acid Cycle (Figure 1.3). This sequence of reactions in which the acetyl portion of acetyl-CoA is degraded to carbon dioxide and hydrogen atoms occurs in the mitochondrial matrix. Only one molecule of ATP is formed during each revolution of the cycle so a net of two ATP/glucose molecule is formed in this fashion (132).

As stated previously, generation of a proton motive force provides the energy to drive the formation of ATP. Almost 90% of the total ATP formed by glucose metabolism is formed during subsequent oxidation of the hydrogen atoms that were released during the earlier stages of glucose degradation. During the breakdown of one glucose molecule, a total of 24 hydrogen atoms are formed. The hydrogen atoms are released in packets of two, and in each instance, the release is catalyzed by a dehydrogenase enzyme. Twenty of the 24 hydrogen atoms are immediately ionized. Both the free hydrogen ion and the hydrogen bound with nicotinamide adenine dinucleotide (NAD^+) subsequently enter into the electron transport chain. The remaining four hydrogen atoms combine with a specific dehydrogenase and pass directly from the dehydrogenase into the oxidative process (132).

Oxidation of hydrogen is accomplished by a series of enzymatically catalyzed reactions that split each hydrogen atom into a hydrogen ion and an electron. The electrons eventually combine oxygen with water to form hydroxyl ions. The hydrogen and hydroxyl ions then combine with each other to form water. During the sequence of oxidative reactions, tremendous quantities of energy are released to form ATP.

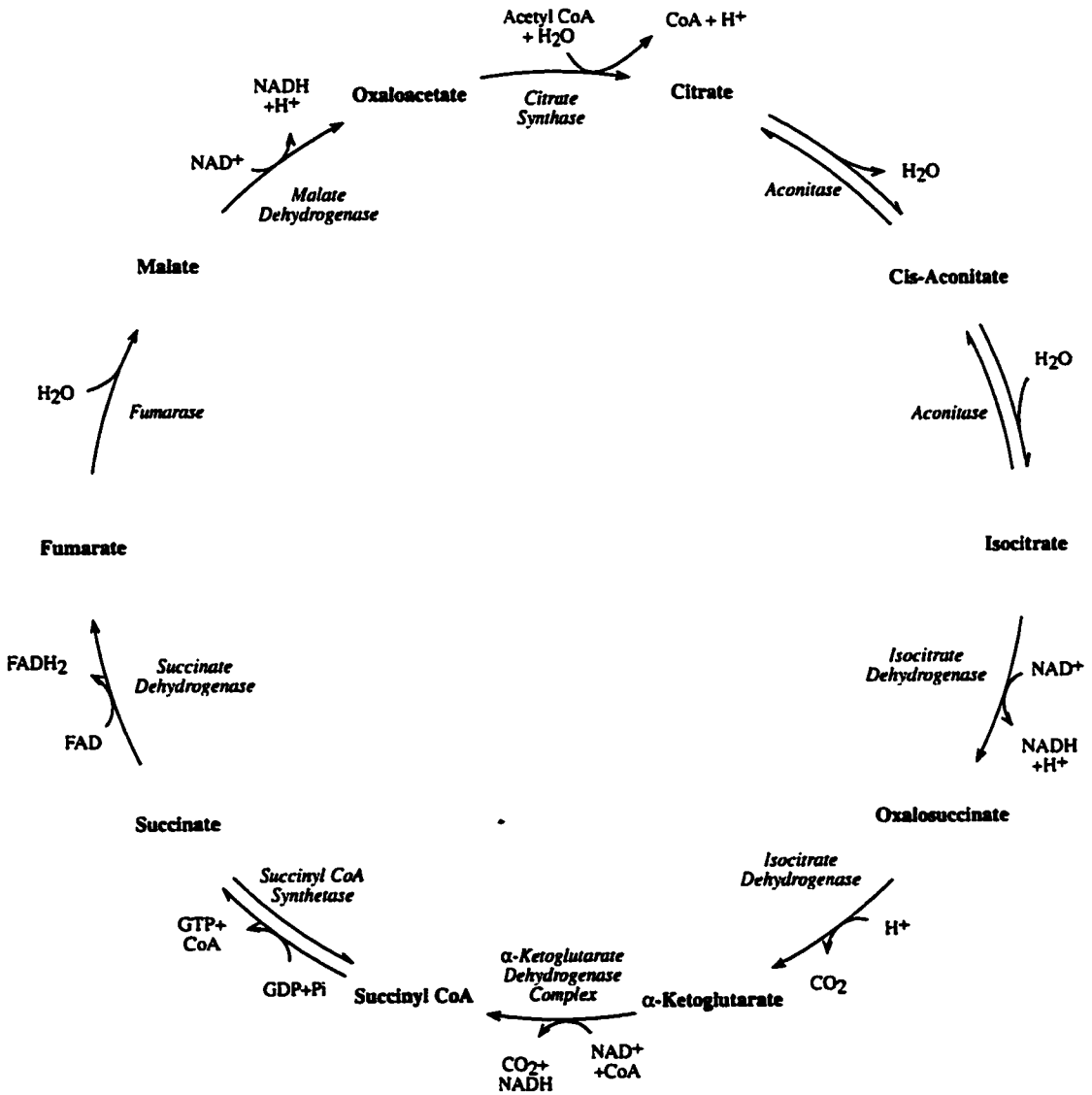


Figure 1.3 - Citric Acid Cycle

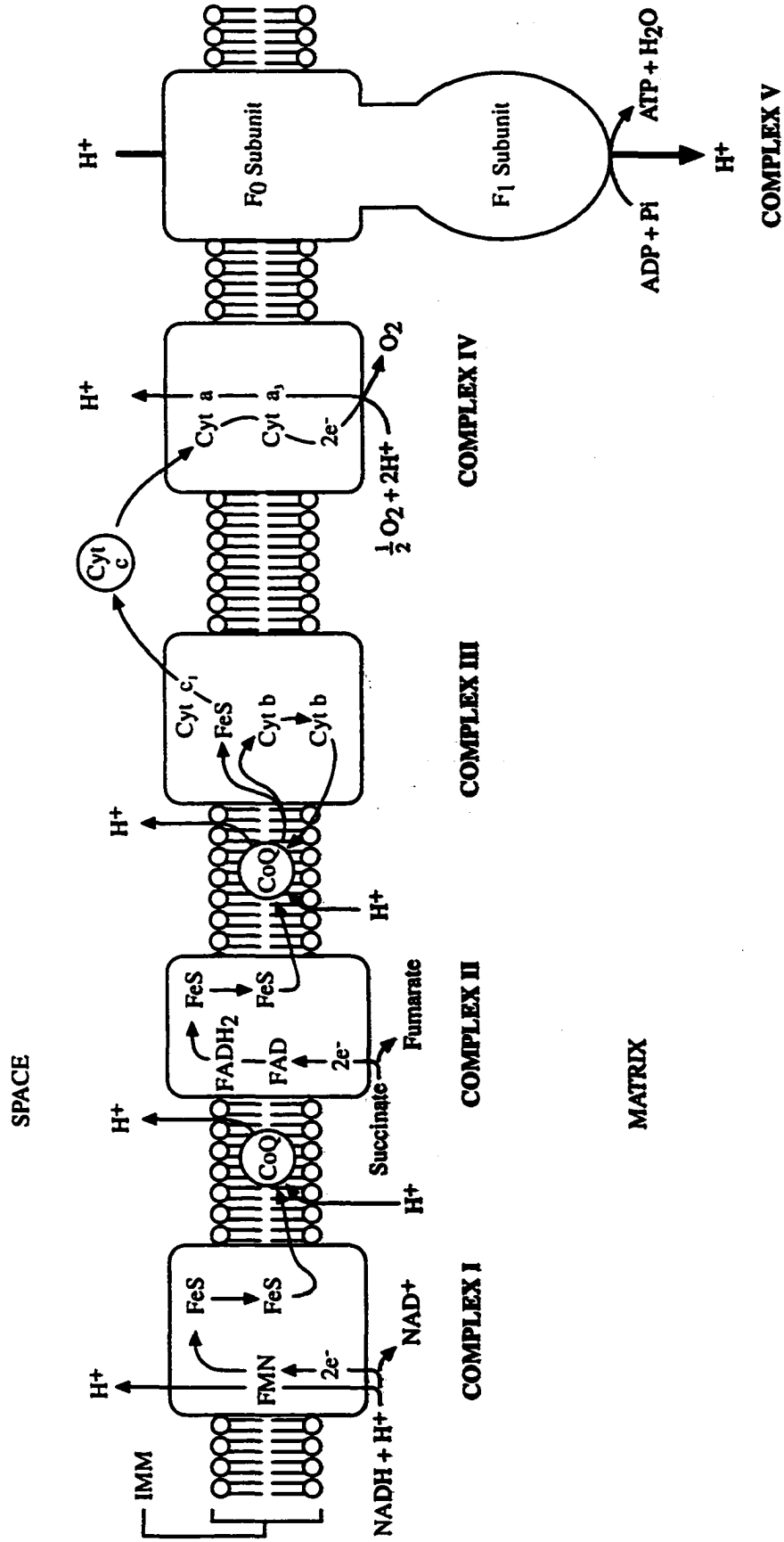
Formation of ATP in this manner is called oxidative phosphorylation, which occurs in the mitochondria by the chemiosmotic mechanism (Figure 1.4) (132).

The electrons immediately enter an electron transport chain of electron acceptors, which include membrane flavoprotein, several iron sulfide proteins, ubiquinone, and cytochromes B, C, C_1, A, and A_3. All are an integral part of the inner mitochondrial membrane. When the electrons reach cytochrome A_3 (cytochrome oxidase), elemental oxygen is reduced to form ionic oxygen, which then combines with hydrogen ions to form water. During transport of these electrons through the electron transport chain, large amounts of energy are released to drive the synthesis of ATP. The energy generated during the chemiosmotic mechanism is used to pump hydrogen ions from the inner mitochondrial membrane into the outer chamber between the inner and outer mitochondrial membranes. This creates a high concentration of positively charged hydrogen ions in the outer chamber and a strong negative electrical potential in the inner mitochondrial matrix (132).

The next step is the conversion of ADP to ATP. This process involves ATP synthetase, a large protein molecule that protrudes through the inner mitochondrial membrane and into the inner matrix. The high concentration of positively charged hydrogen ions in the outer chamber and the large electrical potential difference across the inner membrane cause the hydrogen ions to flow into the mitochondrial matrix through the ATPase molecule. This proton-motive force provides the energy necessary to convert ADP into ATP (132).

The final step in the process is the transfer of the ATP from the mitochondrion to the cytoplasm. This occurs by facilitated diffusion through the inner mitochondrial

Figure 1.4 - Electron transport chain. IMM = inner mitochondrial matrix; FMN = flavoprotein; FeS = iron sulfide proteins; CoQ = ubiquinone; Cyt = cytochrome.



membrane and simple diffusion through the outer mitochondrial membrane. In turn, ADP is transferred in the opposite direction to allow for continual conversion into ATP. For each pair of electrons that pass through the electron transport chain, three ATP molecules are synthesized (132).

In all, 38 molecules of ATP are formed for each molecule of glucose that is degraded to carbon dioxide and water. Thus, 456,000 calories of energy can be stored in the form of ATP, whereas 686,000 calories are released during the complete oxidation of one molecule of glucose. The overall efficiency of energy transfer is 66%, with the remaining 34% of energy being lost in the form of heat (132).

1.5 Purines and Purinergic Receptors

Extracellular purines (ATP, ADP, AMP and adenosine) (Figure 1.1) are important signaling molecules that are involved in numerous biological processes, including smooth muscle contraction and relaxation, neurotransmission, exocrine and endocrine secretion, the immune response, inflammation, platelet aggregration, pain, and modulation of cardiac function (133). In 1929, Drury and Szent-Györgyi suggested that purines may serve as extracellular signaling molecules based on their studies which demonstrated that adenosine and AMP extracted from heart muscle could induce heart block, arterial dilatation, hypotension, and inhibition of intestinal smooth muscle contraction (134). Gillespie, in 1934, demonstrated that the potency and response to extracellular purines was influenced by the presence or absence of the phosphate units of adenine nucleotides (135). Phosphate removal increased the ability of adenine compounds to cause vasodilatation and hypotension, whereas ATP, which contains 3 phosphate units, increased blood pressure in the cat and rabbit that was rarely or never

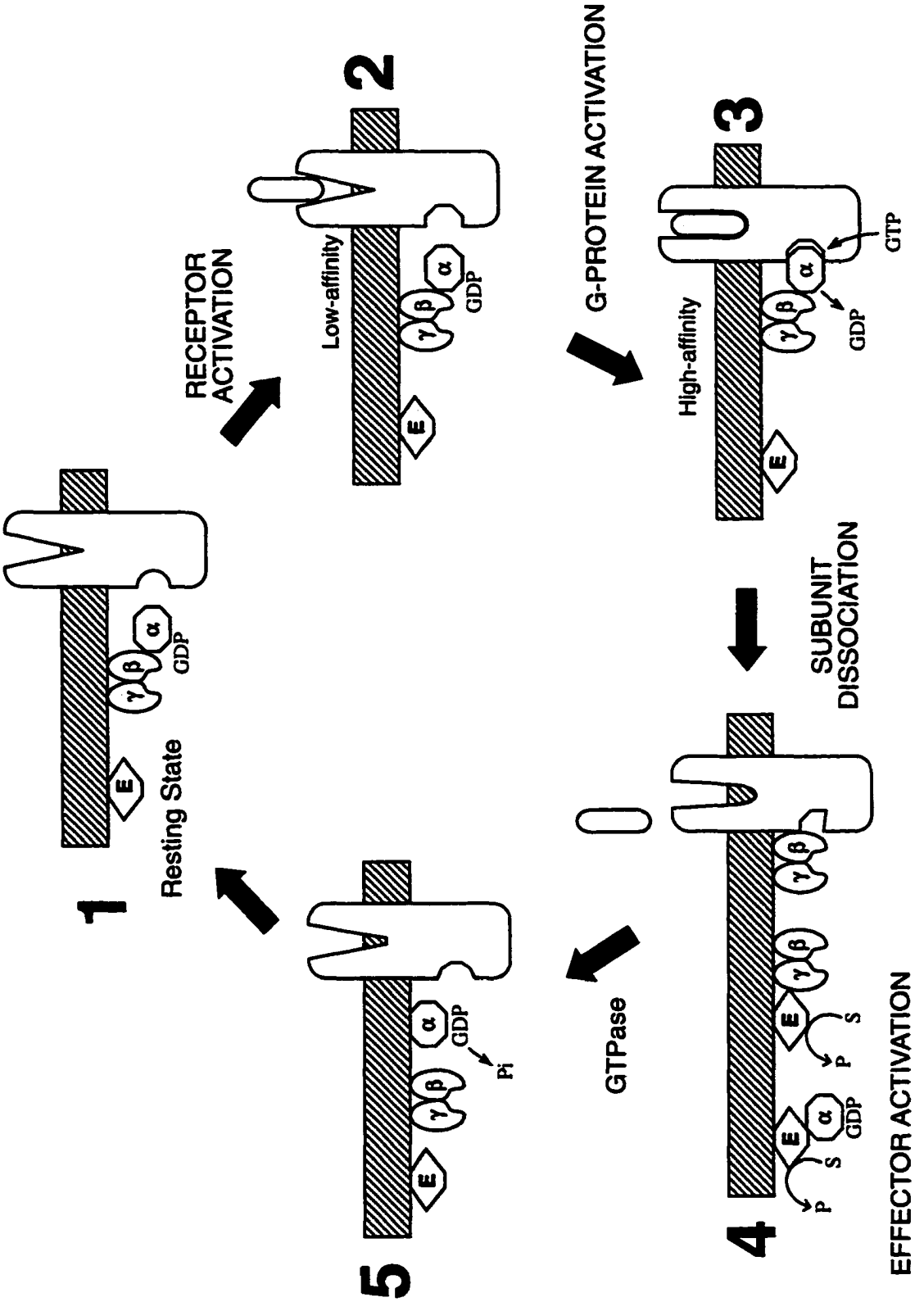
observed with AMP or adenosine. Furthermore, ATP was shown to be more potent than AMP and adenosine in causing contraction of the guinea pig ileum and uterus smooth muscle (135). These results supported the concept that different purine receptors exist and that different adenine nucleotides have different mechanisms of action.

"Purinergic" receptors were first formally recognized by Burnstock in 1978 and were divided into two main categories: "P_1-purinoceptors", which adenosine is the principal natural ligand, and "P_2-purinoceptors", which recognize ATP and ADP (136). The division was based on several criteria including relative potencies of the adenine nucleotides; selective antagonism of the effects of adenosine by methylxanthines; activation of adenylate cyclase by adenosine; and stimulation of prostaglandin synthesis by ATP and ADP.

Adenosine/P1 and P2Y receptors couple to G proteins. G-protein receptors, which constitute the largest category of receptors, have a conserved structure consisting of seven transmembrane (TM) domains of hydrophobic amino acids. The N-terminal is on the extracellular side and the C-terminal is on the cytoplasmic side of the cell membrane. The TM domains form a pocket where the ligand binding site is located. Purinergic agonists are believed to bind within the upper half of this pocket. Three extracellular and three cytoplasmic hydrophilic loops connect the TM domains. Following ligand binding to the receptor, the intracellular segment of the receptor interacts with the appropriate G protein to activate specific intracellular signal transduction pathways (Figure 1.5) (133).

The TM regions are generally highly conserved and are crucial for ligand binding and specificity (133). Specifically, the carboxyl region of the second EC loop is

Figure 1.5 - G-Protein receptors and their signal transduction mechanism. $\gamma\beta\alpha$ = G protein heterotrimer; GDP = guanosine diphosphate; GTP - guanosine triphosphate; E = effector; S = substrate; P = product; Pi = orthophosphate.



primarily involved in ligand recognition (137,138) and the histidine residues in TM6 and TM7 in ligand binding (138).

To date, four subtypes of adenosine/P1 receptors have been identified: A_1, A_{2A}, A_{2B}, and A_3 (139). The predominant adenosine/P1 receptor type on blood vessels are the A_{2A} and A_{2B} receptors, which are located on the smooth muscle and endothelium. Ligand binding with subsequent receptor activation in the vasculature produces vasodilatation (133). The A_{2A} receptors have a wide-ranging but restricted distribution that includes immune tissue, platelets, the central nervous system (CNS) and vascular smooth muscle and endothelium (133). The most commonly recognized signal transduction mechanism for the A_{2A} receptors is activation of adenylate cyclase via coupling with the G protein, G_s. The receptor/protein coupling is tight; therefore, agonist dissociation is slow (140). Some A_{2A} receptors may activate K_{ATP} channels, which may involve a cAMP-dependent protein kinase (PK) (141). Short-term desensitization of the A_{2A} receptor involves receptor phosphorylation with subsequent dissociation of the receptor from the G protein. Long-term desensitization involves inhibition of adenylate cyclase activity, down-regulation of receptor number, or up-regulation of inhibitory G proteins (G_i) (142-144).

The other major adenosine/P1 receptor subtype associated with blood vessels is the A_{2B}. This receptor couples to different signaling pathways including activation of adenylate cyclase, G_q/G_{11}-mediated coupling to PLC and inositol 1,4,5-triphosphate (IP_3)-dependent increase in Ca^{2+}, and coupling to PLC (145,146). Owing to the lack of selective antagonists for this receptor subtype, little is known about receptor desensitization; however, it may involve inhibition of adenylate cyclase and receptor

phosphorylation and uncoupling from G proteins (142,147). This receptor subtype is important for mediating vasodilatation in some vessels, whereas the A_{2A} predominates in other vessels (148,149).

Categorization of the P2 receptors has been difficult. Recent discoveries have included: (1) multiple P2X receptors are often co-expressed in different proportions in different tissues; (2) P2X receptors can exist as heteromers or homomers; (3) cations can affect P2X channel activity; (4) 2-methylthio ATP, which was previously regarded as a selective P2Y agonist, additionally has profound effects on P2X receptors also; (5) ecto-nucleotidases can affect agonist potencies; (6) antagonists that were previously thought as P2 receptor blockers are non-selective. The lack of selective agonists and antagonists, the coexistence of different P2 receptors, and the degradation and interconversion of purines has prompted re-evaluation of previous conclusions about P2 receptors and has markedly hindered advances in P2 receptor characterization (133).

P2X receptors are ATP-gated ion channels which mediate rapid and selective permeability to cations (Na^+, K^+ and Ca^{2+}) (150-152). To date, seven mammalian P2X receptors ($P2X_{1-7}$) have been cloned and characterized (133). The most significant P2X subtype on vascular smooth muscle is the $P2X_1$ homomer (153).

Ligand binding to the P2X receptor results in the rapid, non-selective passage of cations (Na^+, K^+ and Ca^+) across the cell membrane resulting in an increase in intracellular Ca^{2+} and depolarization (150,151). Membrane depolarization subsequently activates voltage-dependent Ca^{2+} channels, which further contributes to the increase in intracellular Ca^{2+}. Since this pathway does not involve a second-messenger system, the response time is rapid. The concentrations of extracellular cations can markedly

influence the receptor's response since it is a ligand-gated ion channel (133). Desensitization of $P2X_1$ receptor is rapid and will terminate the purinergic response (133). The mechanism of desensitization is not well understood but may involve the hydrophobic domains of the $P2X_1$ receptor (154).

P2X receptors are involved in the generation of excitatory junction potentials (EJP), depolarization, and constriction (155,156). Sympathetic stimulation of blood vessels leads to the development of a rapid EJP that can be blocked by P2 receptor antagonists and desensitization of the $P2X_1$-like receptor. Prolonged periods of stimulation cause summation of the EJPs and the membrane depolarization, which allows opening of voltage-dependent Ca^{2+} channels, Ca^{2+} entry, and contraction (155,157).

P2Y purine receptors couple to G proteins. Currently, they include five mammalian cloned receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) and the uncloned $P2Y_{ADP}$. P2Y receptors are 308-377 amino acid proteins with a mass of 41 to 53 kDa after glycosylation. The seven TM domains are common to that of other G protein-coupled receptors (133).

Coupling of most P2Y receptors to the $G_{q/11}$ protein activates PLC, which stimulates membrane phosphoinositide metabolism and increased production of IP_3 and diacylglycerol (DAG) and mobilization of intracellular Ca^{2+}. Coupling to adenylate cyclase, which involves G_i proteins, by some P2Y receptors has also been described. Since activation of this receptor leads to generation of second messengers, the response time is longer (133).

The $P2Y_1$ and $P2Y_2$ receptors are the most prevalent P2Y receptors located on blood vessels. Other P2Y receptors that have been identified on blood vessels but play only minor roles in the control of vascular tone include the $P2Y_4$ and $P2Y_6$ (133). The $P2Y_1$ receptor is activated by ADP, ATP and certain diadenosine polyphosphates. The receptor exhibits heterogeneity which is most likely attributable to small differences in the structure of the receptor. There is only a sequence homology of 84% between the turkey and human $P2Y_1$ receptor (158). The heterogeneity in ligand binding includes both agonists and antagonists (133). The receptor appears to be more sensitive to adenine nucleotide diphosphates than to triphosphates, and the sensivity to ATP is variable (159-161). Additionally, the electrical charge of the molecule can influence the potency of agonists (162,163).

The principal signal transduction pathway involves activation of PLC. Formation of IP_3 and mobilization of Ca^{2+} stimulates a variety of signaling pathways, including PKC, PLA_2, Ca^{2+}-dependent K^+ channels, nitric oxide synthase (NOS) with subsequent formation of NO, and generation of endothelium-derived hyperpolarizing factor (EDHF). The main physiological target of DAG is stimulation of PKC, which in turn may stimulate phosphatidyl choline-specific PLC, PLD, the mitogen-activated protein kinase (MAPK) pathway, and Ca^{2+} influx via voltage-gated Ca^{2+} channels. Generation of PKC and subsequent phosphorylation of MAPK appears to be the pathway by which these receptors on endothelial cells mediate prostacyclin production (164,165). A second signaling pathway may be inhibition of adenylate cyclase (166). These two pathways are expressed independently (166).

In general, $P2Y_1$ receptors do not readily desensitize. However, when desensitization does occur, it may involve phosphorylation of the receptor by protein kinases and uncoupling of the G protein (133).

$P2Y_1$ receptors are widely distributed and have been described in heart, vascular, connective, immune and neural tissues (133). Their distribution on vascular endothelial and smooth muscle cells implies a role in the regulation of vascular tone. In most blood vessels, $P2Y_1$-like receptors are located primarily on the endothelium and mediate vasodilatation by Ca^{2+}-dependent activation of endothelial NOS with subsequent generation of NO and by generation of EDRF. Endothelial prostacyclin synthesis and release is also stimulated, but appears to play a minor role in the relaxation response (167).

$P2Y_1$ receptors are also present on the smooth muscle of a number of blood vessels and mediate vasodilatation (168-170). The pathway involved in the relaxation response is not known but may involve activation of K^+ channels (171). The significance of these receptors on vascular smooth muscle may be in mediation of non-adrenergic non-cholinergic (NANC) relaxation. ATP released as a neurotransmitter from sensory-motor nerves may activate $P2Y_1$ receptors on the vascular smooth muscle leading to relaxation (170,172,173).

The $P2Y_2$ receptor is activated by ATP and is insensitive or only weakly sensitive to ADP (133). Similar to $P2Y_1$, the $P2Y_2$ leads to phosphoinositide metabolism and Ca^{2+} mobilization but via PLCβ (151). IP_3 formation and Ca^{2+} mobilization stimulate the same signaling pathways as the $P2Y_1$ receptors (133).

Another reported signal transduction pathway involves PLD and stimulation of phosphatidylcholine breakdown (174,175). The mechanism of activation of PLD is unclear but may involve the combined actions of PKC, Ca^{2+}, and G proteins (175). The major route for prostacyclin production in endothelial cells involves protein tyrosine phosphorylation and MAPK activation, which occurs subsequent to activation of PKC but does not involve IP_3 or cytosolic Ca^{2+} (164,165).

$P2Y_2$ receptors are widely distributed and ATP is the natural ligand (133). In the vasculature, they are generally present on the endothelium. Synthesis and release of prostacyclin (PGI_2) and NO results in vascular relaxation (160,176-178). Similar to $P2Y_1$ receptors, $P2Y_2$ receptors do not readily desensitize. Tachyphylaxis has been shown to occur and may involve phosphorylation of the intracellular regions of the receptor (179).

In general, many cells express more than one P2 receptor type. Receptor co-expression permits potential regulation of multiple effectors, fine tuning of agonist-evoked responses, and/or synergy. For example, $P2X_1$ and $P2Y_1$ receptors co-exist on the smooth muscle in some vessels where they may reciprocally control vascular tone by acting as mediators of vasoconstriction and vasodilatation, respectively. Patterns of co-expression may be altered under different physiological and pathophysiological conditions (133).

The local control of vascular tone is regulated by the integrated effects of Adenosine/P1 and P2 receptors (155). For example, $P2Y_1$, $P2Y_2$, A_{2A} and A_{2B} receptors are located on endothelial cells while A_1 and $P2X_1$ receptors are on smooth muscle cells. Normal patterns of purinergic signaling may alter dramatically under pathophysiological

conditions. The net effect of purine receptors may be vasodilatation if endothelial cells are intact, but vasoconstriction may predominate if the endothelium is damaged. In addition, the metabolic relationship between purines, where ATP is catabolized to ADP and adenosine, has important implications for co-localized adenosine/P1 and P2 receptors as there may be an interplay between these receptors (133).

1.6 ATP and ATP-$MgCl_2$

Depletion of ATP in shocked animals was first noted in 1945 by McShan and co-workers (180). During shock, low-flow conditions and organ ischemia, anaerobic glycolysis is activated, which is an inefficient system for the synthesis of ATP (179). During anaerobic glycolysis, ATP utilization exceeds production, resulting in decreased intracellular ATP concentrations (181). If oxygen is not available, pyruvate is unable to follow its normal pathway via acetyl coenzyme A into the Citric Acid Cycle, which results in accumulation of lactic acid within the cell (182). This block in the glycolytic pathway leads to a tendency for glucose to exit the cell, which further reduces substrate availability for ATP production (183).

Under basal metabolic conditions, low levels of ATP are released from cells into the extracellular space. However, when hypoxic conditions exist, the quantity of ATP released is appreciably increased. Upon reoxygenation, ATP release returns to basal levels (184). When myocardium is rendered hypoxic, the large quantities of ATP released can cause profound vasodilatory effects, with subsequent alterations in hemodynamic status (185). Additionally, skeletal and cardiac muscle and brain tissue have been shown to release substantial quantities of ATP into the extracellular space, and these concentrations can profoundly affect vasomotor tone and local blood flow

(183-187). During shock, progressive failure of vital organs occurs, with cardiac failure frequently being the terminal event. Cell membranes begin to depolarize, which leads to intracellular accumulation of sodium and water and decreased intracellular potassium (187-191). These electrolyte/water alterations are suggestive of disruption of the cell membrane and inactivation of the Na^+, K^+-ATPase pump. Depletion of intracellular energy stores, namely ATP, with subsequent inhibition of ATP-dependent cellular responses may occur as a result of alterations in cell membrane transport that occurs during shock (192,193).

Both oxygen and substrate deprivation prevent cells from regenerating ATP, which is required to maintain ionic gradients and structural integrity. Since shock and ischemia can deplete intracellular stores of ATP, the use of exogenously administered ATP to maintain energy stores for cellular metabolism and survival has been investigated. Extracellular ATP can influence many biological processes. When ATP is applied externally to a cell, profound changes in permeability are observed in monolayer cell cultures (194). Upon depletion of intracellular stores of ATP, a marked increase in the sensitivity of cells to external ATP occurs (195). When low concentrations of ATP are added to culture medium, a profound change in cellular permeability of monolayer cell cultures is observed (194). In cell culture, exogenous ATP decreases leakage of intracellular enzymes from human white blood cells and rat lymphocytes (196). This protective effect of ATP appears to be concentration-dependent. At physiological concentrations, the action of ATP is slight and relatively short-lived. However, when higher concentrations are used, the membrane-stabilizing effects are more pronounced and the response persists for a longer period (196).

During shock, administration of ATP has been shown to be protective (197-201). The protective effects of ATP may be due to: myocardial support; replacement of deficient high-energy phosphate stores; and metabolic support by bypassing mitochondrial deficits in electron transport-phosphorylation system (197-200).

During hemorrhage, cellular ATP content is markedly reduced. Administration of ATP during hemorrhagic shock has been shown to improve survival and increase ATP content of heart and liver compared with untreated rats subjected to hemorrhage. When ATP was administered prior to hemorrhage, survival rate was 80% compared with 30% in control animals. If ATP was administered after hemorrhage, survival decreased to 60%. The beneficial effects of ATP on survival following hemorrhage was not due solely to its vasodilatatory action, since other hypotensive drugs failed to be protective. Since administration of pyrophosphate and AMP was not protective, the beneficial effects of ATP may be due to the terminal high energy phosphate bond (197).

Glucose homeostasis and insulin dynamics play a critical role in the pathogenesis of endotoxin shock (202). During experimentally-induced endotoxic shock, both dogs and rats develop hypoglycemia and hyperinsulinemia (203,204). Phosphorylation of cell membranes occurs with administration of ATP, which markedly depresses insulin-stimulated glucose transport and metabolism (205). Administration of ATP during experimentally-induced endotoxic shock in rats depressed the hypercatabolism of glucose that occurs (201).

Both vascular endothelial and cardiac smooth muscle cells contain high levels of ecto-ATPases on their membrane surface. The loss of intracellular ATP during ischemia may be partially mediated through these enzymes. One proposed mechanism

to explain the beneficial effects of exogenous ATP may be that ATP binds receptors involved in activation of ectoenzymes and blocks them, thereby sparing intracellular adenine nucleotides from degradation (206).

Another proposed mechanism may involve direct entrance of ATP, or other adenine nucleotides, into the cell to restore depleted energy stores. In isolated canine hearts, the inability of myocardial cells to regenerate ATP may be due to loss of nucleotide precursors through metabolism of these substances to inosine, with subsequent loss of inosine to the extracellular space (207). Addition of exogenous ATP, but not ADP or AMP, was found to increase the ATP and total adenine nucleotide content of hypoxic myocardium (208).

Substantial evidence that exogenous ATP can influence many biological functions has existed for a long time, but whether ATP exerts its effects by gaining access to the intracellular compartment is not known. In studies utilizing isolated skeletal muscle preparations, exogenous ATP induced muscle contraction (209). However, whether the response was due to a surface effect or an intracellular effect was not known. In 1970, ^{14}C-ATP was shown to enter intact skeletal muscle cells in vitro and the labeled intracellular ATP was due to transport of ATP itself and not due to formation of ATP from its breakdown products (210). Further studies have demonstrated that both ATP and ADP can enter the cell but that AMP remains in the extracellular compartment (211). Evidence to support this claim was obtained by use of ADP, which decreased the ATP:ADP ratio. This was further substantiated with an ATP regenerating system, which increased the ATP:ADP ratio (210). However, whether ATP is intracellular or is binding to specific membrane sites and concentrating on the

surface remains unknown. Surface binding is unlikely due to extensive degradation of ATP in the tissues; the volume of distribution of ADP is less than ATP; and external AMP remains extracellular (210,212). Substantial evidence supports the claim that ATP can enter the cell membrane of tissues (213,214) and that the process is enhanced during adverse circulatory conditions (215,216). Studies also provide evidence for intracellular uptake of ATP (217).

The mechanism of how ATP enters the cell is not understood but may involve a carrier-mediated transport system (212,218). Small quantities of exogenous ATP (1 mM), but not ADP or AMP, increases ATP and total adenine nucleotide content in hypoxic myocardium (208). Furthermore, addition of ATP, but not ADP or adenosine, to culture medium increased ATP content of cultured myocardial cells (219). There is some evidence to support the concept that ATP translocation is associated with a Na^+, K^+-ATPase (220).

Inside of the cell, both ATP and ADP complex with magnesium (221). Most ATP reactions require ATP as a substrate and magnesium as a cofactor (221). Therefore, the reported beneficial effects of exogenous ATP may be enhanced with addition of magnesium. A complex of ATP-$MgCl_2$ has been shown to be capable of entering intact cells (210). A study in rabbits showed that injected ATP-$MgCl_2$ is retained in the circulation and is present in sufficient qunatities to provide beneficial effects to cells (222). When ATP was combined with magnesium-chloride ($MgCl_2$), the effect of ATP on cellular function and microcirculatory blood flow during shock and ischemia was enhanced (206). Beneficial effects were not observed if ATP or $MgCl_2$ alone or adenosine-$MgCl_2$ were infused (206). ATP is a biological complexing agent

and if given alone, may chelate divalent cations in the vascular system and cause different hemodynamic effects than those that are observed when ATP is complexed with $MgCl_2$ (221).

The mechanism of how $MgCl_2$ enhances the effect of ATP is unknown; however, it has been shown both in vivo and in vitro that magnesium may inhibit deamination and dephosphorylation of ATP by tissues (221) and prevent the ATP from coupling with other ions (223). Thus, if ATP is administered in conjunction with $MgCl_2$, a higher concentration of ATP could be available for the tissues to utilize (221). In addition, $MgCl_2$ may help replenish cellular magnesium levels that are decreased during shock states (206,224) and enhance several important reactions that involve ATP (225). Tissue and mitochondrial magnesium levels have been shown to significantly decrease following ischemia and reperfusion and ATP-$MgCl_2$, but not ATP or $MgCl_2$ alone, substantially improves tissue and mitochondrial magnesium levels (221).

The hemodynamic alterations associated with administration of ATP-$MgCl_2$ have been studied in both normal and pathophysiological situations. In man, ATP-$MgCl_2$ (0.1-0.4 mg/kg/min) increased CO by 76%, principally by increasing heart rate (43%). However, stroke volume index was also increased (14%). The increase in CO was correlated with rate of infusion (0.1-0.4 mg/kg/min), not the total dose. Changes in MAP were not observed in the study. At the highest infusion rate (0.4 mg/kg/min), SR_L decreased (226).

During ischemia, oxygen and substrate deprivation leads to a decrease in cellular ATP production. Since ATP is necessary to maintain ionic gradients and structural integrity, cellular damage ensues (227). Utilizing intravital fluorescence microscopy,

Clemens and colleagues demonstrated that administration of ATP-$MgCl_2$ following hepatic ischemia improved microcirculatory blood flow to the surface of the liver. Specifically, the improvement in blood flow was due to a decrease in the loss of perfused capillaries during reperfusion (227). Additionally, leakage of intracellular enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) were reduced (228). In a canine hypothermic heart ischemia/reperfusion model, administration of 1 mg/kg/min ATP and 0.33 mg/kg/min $MgCl_2$ during reperfusion resulted in complete functional recovery, whereas control animals showed marked reduction in hemodynamic performance and myocardial compliance (229). Fedelesova and colleagues demonstrated in isolated, nonperfused hypothermic canine hearts that a portion of exogenous ATP was broken down to ADP and AMP and that some of the ADP entered the cell and became phosphorylated to regenerate ATP (230).

In an isolated perfused rat kidney model, ischemia caused a rapid decline in ATP levels, as assessed with high-resolution ^{31}P-nuclear magnetic resonance spectroscopy (231). Upon reperfusion, a 56% increase in tissue ATP content was observed within 10 min. Thereafter, a slow decline in ATP content occurred, and by 75 min, the ATP content had decreased to approximately 33% of normal values. When the kidney was perfused with 0.3 mM ATP-$MgCl_2$, renal ATP levels increased to 69% of normal values within 10 min, and by 75 min they were normal. Additionally, the intracellular acidosis and decreased blood flow induced by ischemia/reperfusion was reversed. Cessation of blood flow to the kidney leads to a depletion of high-energy phosphate stores and a steady rise in Pi levels. The improvement in renal ATP content may be due to direct entry of ATP into tubular epithelial cells to provide metabolic energy (216) or to a

priming effect on the resynthesis of adenine nucleotides (232) and repletion of the precursor pool (231).

Mesenteric ischemia with subsequent reperfusion results in mucosal barrier dysfunction, manifested by transcellular movement of fluid from the microcirculation and, in the reverse direction, translocation of endotoxin from the bowel lumen to the lymphatic vessels (233) and portal blood (234). Depletion of cellular energy stores (ATP) contributes to the cellular dysfunction (235-238). The effects of ATP-$MgCl_2$ on intestinal permeability, ATP content, and blood flow during ischemia has been evaluated. In a rat model of intestinal ischemia, plasma to lumen clearance of ^{51}Cr-EDTA was used as a marker of altered permeability. Ischemia induced by mesenteric arterial occlusion for 90 min increased permeability, and this was associated with a significant reduction in blood flow as assessed by radiolabeled microspheres. Rats pretreated with ATP-$MgCl_2$ demonstrated no increase in ^{51}Cr-EDTA clearance over 90 min. However, the decrease in blood flow induced by ischemia was not reversed. Tissue ATP levels were reduced within 5 min of ischemia and remained decreased throughout 90 min. Administration of ATP-$MgCl_2$ did not significantly alter tissue ATP levels (239). ATP-$MgCl_2$ improved survival in neonatal rats exposed to intestinal ischemia (240) and improved intestinal mucosal function as assessed by absorptive capacity (241).

In vitro studies using intestinal epithelial cells have demonstrated that hypoxia decreases ATP content in conjunction with increases in paracellular permeability (242). Depletion of ATP produces disruption of cortical actin and actin stress fibers in epithelial cells, which results in a decrease in epithelial monolayer integrity (243). In

the study by Kreienberg and colleagues, the effects of ATP-$MgCl_2$ appear to be through a mechanism other than providing cells with an energy source (239). ATP-$MgCl_2$ may exert a priming effect or it may enter the cell to provide sufficient high-energy phosphates to prevent dephosphorylation of key structural proteins. Improvement in mucosal barrier function may be mediated through activation of P2 receptors and increased cAMP, which subsequently increases junctional integrity of epithelial cells (244). Alternatively, the ATP may degrade to adenosine, bind P1 receptors and increase cAMP to produce a similar effect (245-247).

In a canine hemorrhage shock model, Horton and colleagues demonstrated that dogs administered lactated Ringer's solution and ATP-$MgCl_2$ (100 μmole/kg each) following hemorrhage had lower mean arterial pressure, cardiac output, stroke volume and rate of left ventricular pressure rise compared with dogs given lactated Ringer's solution alone (248). The reduced cardiac performance after ATP-$MgCl_2$ administration occurred despite adequate coronary blood flow and adequate myocardial oxygen delivery. A decreased myocardial oxygen extraction and a negative myocardial lactate balance after intravenous ATP-$MgCl_2$ indicate a cellular metabolic defect (248). These results contradict observations of another report that indicated ATP-$MgCl_2$-glucose improved cardiac filling, stroke volume and CI despite a fall in heart rate (HR) and SR_L (249). Additionally, administration of ATP, regardless of concomittant blood replacement, failed to appreciably alter CO in hemorrhaged dogs (197).

Hypoxia and shock have been found to be potent stimulants of inflammatory cytokine production (250). In a study of hemorrhage and resuscitation in rats, administration of ATP-$MgCl_2$ markedly decreased TNF and IL-6 levels to those

comparable with sham controls. ATP-$MgCl_2$ also restored hepatic blood flow and hepatic function (as assessed using the extraction ratio for indocyanine green), which was decreased with hemorrhage (251). Thus, downregulation of the synthesis and/or release of inflammatory cytokines, TNF and IL-6, may be one of the mechanisms responsible for the beneficial effects observed with ATP-$MgCl_2$ following trauma-hemorrhage and crystalloid resuscitation (251). The agent may appreciably improve microcirculation and thus prevent continued hypoxic insult following ischemia and hemorrhagic shock (252-254). Another potential effect of ATP-$MgCl_2$ on cytokine production results from suppression of the respective gene transcription and/or translation (251). Conversely, the downregulation might be caused by an increase in circulating cytokine receptor or inhibitor levels (255). ATP-$MgCl_2$ could directly or indirectly decrease TNF and IL-6 synthesis by Kupffer cells and consequently prevent the deleterious effects of TNF or IL-6 on hepatocytes (251).

The efficacy of ATP-$MgCl_2$ during sepsis has been evaluated. Using a cecal ligation and puncture model of sepsis in rats, administration of saline, glucose (1g), low-dose ATP-$MgCl_2$ (12.5 μmole each), or low-dose ATP-$MgCl_2$ plus glucose did not alter survival rates. However, when high-dose ATP-$MgCl_2$ (100 μmole ATP and 50 μmole $MgCl_2$) plus glucose was used, a significant increase in survival was observed (256).

Upon induction of sepsis, both hepatic and renal ATP levels were decreased (256). High dose ATP-$MgCl_2$ alone or in conjunction with glucose restored cellular ATP within 3 hours, as determined by spectrophotometric methods. During sepsis, animals develop hypoglycemia. If glucose is administered alone, the cells are unable to utilize it so hyperglycemia develops. If ATP-$MgCl_2$ and glucose are administered, cells

are capable of utilizing glucose (257). Additionally, reticuloendothelial system dysfunction occurred during sepsis, which was restored with administration of high-dose ATP-$MgCl_2$ plus glucose (256).

During Group B streptococcal sepsis in piglets, increases in MAP and PAP, SR_L and PR_L and PR_L:SR_L ratios were observed. Additionally, CO and stroke volume decreased. Administration of a continuous infusion of ATP-$MgCl_2$ (0.6 μmole/kg/min) reversed the hemodynamic alterations that were induced by sepsis. Also, the median survival was longer for the piglets treated with ATP-$MgCl_2$. Finally, lung compliance was higher and pulmonary airway resistance was lower in treated piglets (258).

1.7 Summary of Literature and Hypotheses for Present Studies

During shock, there are alterations in organ blood flow, which affect cell membrane transport and function, energy metabolism, and mitochondrial function. ATP utilization exceeds it production, which results in depletion of intracellular ATP stores. Therefore, a major rate-limiting factor in shock and ischemia, and thus resuscitation, is resynthesis of ATP.

Administration of exogenous ATP, primarily complexed with $MgCl_2$ (ATP-$MgCl_2$) has been demonstrated to decrease morbidity and improve survival in various shock models. The use of ATP-$MgCl_2$ after hemorrhagic shock or other adverse circulatory conditions improves mitochondrial function and tissue ATP content; restores organ function, blood flow, and microcirculation; improves survival time and survival rate; and down-regulates the synthesis and release of inflammatory cytokines.

Adenine nucleotides can alter vasomotor tone through interaction with purinergic receptors located on the vascular endothelium and smooth muscle cells.

Activation of the P2X receptor (predominantly smooth muscle) by ATP opens ATP-gated ion channels, resulting in changes in intracellular Ca^{2+} concentrations with subsequent membrane depolarization and contraction of the smooth muscle. Activation of the P2Y receptor (predominantly on the endothelium) by ATP or the A_2 receptors (smooth muscle) by adenosine results in receptor coupling to G proteins and activation of second messenger systems leading to vasodilatation. Since the predominant vasomotor effect of adenine nucleotides is vasodilatation, administration of exogenous ATP may enhance organ blood flow and microcirculation during shock and ischemia.

In adult horses, acute gastrointestinal tract disease is the leading natural cause of death. Strangulating volvulus of the ascending colon occurs frequently and is associated with a high mortality rate. The volvulus creates colonic luminal obstruction and vascular occlusion, with subsequent colonic ischemia, mucosal necrosis and vascular thrombosis. Despite surgical correction, many horses die; this may be due to a sustained decrease in blood flow and lack of substrate availability for cellular metabolic functions. Additionally, disruption of the mucosal barrier leads to translocation of bacteria and endotoxin into the splanchnic and systemic circulation. If sufficient endotoxin enters the systemic circulation, death can ensue.

Administration of ATP-$MgCl_2$, which has vasodilatory actions, increases CO and delivers energy substrate (ATP) and co-factor (Mg) directly to tissues, may offer a potential therapy for horses with intestinal ischemia, endotoxemia and shock. However, no information is presently available on the effects of ATP-$MgCl_2$ in horses. The hypotheses of the studies presented in this dissertation include:

Study 1 - IV infusion of ATP-$MgCl_2$ will cause rate-dependent alterations in hemodynamic variables with minimal changes in metabolic, hematologic and serum biochemical variables and no detrimental effects in clinically healthy, conscious, adult horses.

Study 2 - IV infusion of ATP-$MgCl_2$ will cause a rate-dependent decrease in systemic and colonic vascular resistance, principally via vasodilatation, in clinically healthy, anesthetized, adult horses.

Study 3 - IV infusion of ATP-$MgCl_2$ will significantly attenuate the pathophysiologic alterations in clinical signs; cardiopulmonary, metabolic, hematologic, and serum biochemical variables; and serum cytokines subsequent to low-dose endotoxin infusion in conscious, adult horses.

Study 4 - The vasomotor tone of isolated equine colonic arterial and venous rings in response to administration of exogenous ATP will be significantly attenuated with endothelium removal and incubation with a non-specific NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME).

Study 5 - An established method for adenine nucleotide quantitation, using high performance liquid chromatography, can be modified and validated for use on equine colonic mucosal tissue.

Study 6 - Using an electron transport inhibitor (antimycin A) and physiologic solution devoid of substrate, equine colonic mucosal adenine nucleotide content can be depleted in vitro and then repleted upon removal of the antimycin A and addition of substrate.

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CHAPTER 2. HEMODYNAMIC AND METABOLIC ALTERATIONS ASSOCIATED WITH INTRAVENOUS INFUSION OF A COMBINATION OF ADENOSINE TRIPHOSPHATE AND MAGNESIUM CHLORIDE IN CONSCIOUS HORSES*

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2.1 Introduction

Shock can be defined as "inadequate blood flow to vital organs or the inability of the body cell mass to metabolize nutrients normally" (1). The predominant changes during shock develop in the microcirculation, affecting cell membrane transport and function, energy metabolism, and mitochondrial function (2). During hypoxic conditions, endogenous production of ATP is decreased (3). A major rate-limiting factor in shock and ischemia, and thus resuscitation, is resynthesis of ATP (4). Therefore, a logical therapeutic approach to increase tissue ATP concentrations is to infuse the substrate (ATP) directly, rather than administer agents that would lead to ATP synthesis (5).

Administration of a combination of ATP and $MgCl_2$ in humans results in peripheral vasodilatation and increase in cardiac output (6). These findings suggest its potential beneficial use in patients with hypoperfusion (low-flow) or organ ischemia. Use of an ATP-$MgCl_2$ combination after hemorrhagic shock and other adverse circulatory conditions in humans and laboratory animals improves mitochondrial function and tissue ATP content (2,7); restores organ function, blood flow, and microcirculation (7-12); improves reticuloendothelial function, survival time, and survival rate (13,14); and down-regulates synthesis and release of inflammatory cytokines (15).

Low-flow conditions and organ ischemia develop commonly in horses subsequent to intestinal strangulation, enterocolitis and proximal enteritis, laminitis, endotoxemia, sepsis, and severe dehydration and exhaustion (16). Anaerobic glycolysis is activated during ischemia but is an inefficient system for production of ATP (17).

During anaerobic glycolysis, ATP utilization exceeds production, resulting in decreased ATP concentrations (17). If oxygen is not available, pyruvate is unable to follow its normal pathway via acetyl coenzyme A into the Krebs-citric acid cycle, which results in accumulation of lactic acid within the cell (18). This block in the glycolytic pathway leads to a tendency for glucose to exit the cell, which further reduces substrate availability for ATP production (19). If ATP-requiring processes increase to maintain cellular integrity and function in the presence of reduced ATP production, a further oxygen deficit would be expected (17).

The purposes of the study reported here were to determine the hemodynamic and metabolic effects of IV infusion of ATP-$MgCl_2$ combination in clinically normal, conscious adult horses, and to determine a maximal safe IV infusion rate.

2.2 Materials and Methods

2.2.1 Horses — The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Six clinically normal female horses (4 Thoroughbreds and 2 Quarter Horses), ranging in age from 3 to 13 (median, 10.5) years old and weighing from 439 to 549 kg (median, 490 kg), were studied. Horses were maintained on a routine preventive health care program and were vaccinated and dewormed 2 weeks prior to the study. All horses were kept on pasture and conditioned to stand in the study area. On the day of study, horses were placed in a research stall (1.82 x 1.82 m), and crossed tied. Hay and water were provided ad libitum.

2.2.2 Instrumentation — Horses were instrumented, using described techniques (20). All catheters were placed percutaneously after aseptic preparation of the skin and SC infiltration of lidocaine. A 14-gauge, 13.3-cm Teflon catheter[a] was

inserted into the left jugular vein for infusion of the ATP-$MgCl_2$ combination. A 14-gauge, 5.1-cm Teflon catheter[b] was inserted proximal to the first catheter for collection of jugular venous blood. A balloon-tipped, flow-directed thermodilution catheter,[c] which was used for measurement of cardiac output (CO) and pulmonary artery pressures (PAP), was inserted into the right jugular vein and advanced until the distal port was positioned in the pulmonary artery. Polyethylene tubing[d] (outside diameter [OD], 1.77 mm) was inserted into the right jugular vein proximal to the thermodilution catheter and advanced until the tip was positioned in the right ventricle for infusion of ice-cold polyionic fluids[e] for measurement of CO. A 55-ml volume of fluid was infused over 4 seconds into the right ventricle, using a carbon dioxide-driven injector,[f] and the CO was derived on the basis of thermodilution (21). The CO meter[g] was connected to a polygraph,[h] and CO curves were generated and recorded on a chart recorder.[i] Arterial blood pressures were measured by use of a 20-gauge, 5.1-cm Teflon catheter[j] placed in the transverse facial or facial artery. All catheter positions were confirmed by evidence of characteristic pressure wave forms. All pressures (systemic and pulmonary artery) were measured, using a pressure monitor[k] with the transducer positioned at the level of the point of the shoulder. A silicone catheter[l] (OD, 12 mm) with a balloon was placed securely in the urinary bladder for urine collection. A continuous base-apex ECG[k] also was obtained.

2.2.3 The ATP-$MgCl_2$ formulation — The formulation of ATP-$MgCl_2$ has been described (22). On the basis of 450 kg of body weight, 100 μmole of ATP/kg[m] (27.225 g) and 100 μmole of $MgCl_2$/kg[n] (9.1485 g) were weighed and placed in separate sterile beakers. Sterile water (50 ml) was added to the ATP and stirred until the ATP

was completely dissolved. The pH of the ATP solution was adjusted to 6.5, by addition of 5*N* sodium hydroxide (NaOH), then to 7.0, using 1*N* NaOH, and to final pH of 7.4, using 0.1*N* NaOH. It is extremely important not to exceed a pH of 7.4 because the ATP will be degraded (22). Sterile water (100 ml) was added to the $MgCl_2$, and the mixture was stirred until the $MgCl_2$ dissolved. The $MgCl_2$ solution was then slowly added to the ATP solution. The pH was adjusted again to 7.4, using 1*N* initially then 0.1*N* NaOH. The ATP-$MgCl_2$ solution was passed through a 0.22-μm filtration unit[o] and stored at 4 C until used. To calculate appropriate infusion rates for each horse, the ATP-$MgCl_2$ solution (200 ml) was added to saline (0.9% NaCl) solution (800 ml) to produce a final concentration of 27.225 mg of ATP/ml of solution.

2.2.4 Experimental design — All horses received an IV infusion of the ATP-$MgCl_2$ combination via an infusion pump,[p] beginning at a rate of 0.05 mg of ATP/kg of body weight/min. The infusion rate was increased by 0.05 mg/kg/min increments at 10-minute intervals until a maximal rate of 1.0 mg/kg/min (maximum volume of 16.65 ml/horse/min) was achieved. Unless otherwise stated, data were collected prior to the start of the infusion (time = 0), at the end of each infusion rate, and at 15-minute intervals for the next hour after discontinuation of the infusion. Because hemodynamic effects have been documented to be dependent on the rate of infusion rather than the dose of ATP-$MgCl_2$ preparation (Chaudry, 1982b), baseline data (time = 0) served as a control for each horse.

2.2.5 Clinical signs of disease — Heart rate (beats/min), respiratory rate (breaths/min), mucous membrane color, capillary refill time (seconds), rectal temperature (°C), gastrointestinal borborygmi, and behavior were monitored. Rectal

temperature was obtained, using a mercury-containing thermometer. Gastrointestinal borborygmi were assessed subjectively (increased, normal, decreased, or absent) by the same investigator by auscultation (30 s/quadrant) of the abdominal cavity in 4 quadrants (right dorsal, right ventral, left dorsal, and left ventral). Specific behavioral alterations were recorded.

2.2.6 Hemodynamic variables — Hemodynamic variables that were measured included systolic, diastolic, and mean systemic and pulmonary arterial pressures (SAP, DAP, MAP and SPAP, DPAP, MPAP, respectively; mm Hg) and CO (L/min). Three measurements were taken at each time for each pulmonary and facial arterial pressure. Five measurements were taken for CO at each time, and the 3 middle values were used for analysis. Cardiac index (CI; CO ÷ kg of body weight; ml/min/kg), stroke volume (SV; CO ÷ heart rate [HR]; L/beat), systemic vascular resistance (SR_L; MAP ÷ CO; mm Hg/L/min), and pulmonary vascular resistance (PR_L; MPAP ÷ CO; mm Hg/L/min) were calculated (23). Specific ECG alterations were recorded.

2.2.7 Metabolic variables — Facial arterial blood samples (2 ml each) were anaerobically collected into separate heparinized syringes and stored on ice until analyzed[q] for pH, partial pressure of carbon dioxide ($PaCO_2$; mm Hg), partial pressure of oxygen (PaO_2; mm Hg), percentage oxygen saturation (SaO_2; %), bicarbonate concentration (HCO_3^-; mEq/L), total CO_2 (TCO_2; mmol/L) and base excess. All samples were analyzed within 1 hour of collection. Systemic arterial oxygen content (CaO_2; ml/dl) was calculated as the sum of oxygen bound to hemoglobin (Hb) and oxygen dissolved in plasma ([Hb x $\%SaO_2$ x 1.34] + [PaO_2 x 0.003]) (24). Oxygen delivery (DO_2; ml/min) was estimated as the product of CaO_2 and CO ($DO_2 = CaO_2$ x CO) (24).

Since Hb concentrations did not significantly change across time for any horse in the study, each horse's baseline Hb value was used in the calculations of CaO_2.

2.2.8 Hematologic variables — Jugular venous blood (3 ml) was collected into tubes containing EDTA for analysis of PCV (%) and total solids concentration (g/dl). Complete blood count and fibrinogen concentration were determined[r] prior to the start of the infusion, at 1.0 mg/kg/min infusion rate, and 1 hour after discontinuation of the infusion. Red and WBC indices also were determined at 2, 6, and 24 hours after discontinuation of the infusion.

2.2.9 Serum biochemical variables — Jugular venous blood (6 ml) was collected into tubes containing lithium heparin and was analyzed[s] for sodium (mmol/L), potassium (mmol/L), chloride (mmol/L), phosphorus (mg/dl), calcium (mg/dl), total protein (g/dl), albumin (g/dl), globulin (g/dl), BUN (mg/dl), creatinine (mg/dl), glucose (mg/dl), aspartate transaminase (AST; U/L), γ-glutamyl transferase (GGT; U/L), alkaline phosphatase (ALP; U/L), total bilirubin (mg/dl), creatine kinase (CK; U/L), TCO_2 (mmol/L), and anion gap (mmol/L). Samples were collected prior to the start of the infusion, at 1.0 mg/kg/min infusion rate, and 1 hour after discontinuation of the infusion.

2.2.10 Urine output — The urinary bladder was emptied, urine volume (ml) was quantified, and specific gravity was determined.

2.2.11 Statistical analyses — All data were considered continuous and evaluated for normality, using the Shapiro-Wilk statistic. Data were considered to follow a normal distribution, with failure to reject the null hypothesis of normality at $P \leq 0.05$. Normal data were summarized and graphed as mean ± SEM.

All quantitative data were analyzed, using the model $y = u + \text{horse} + \text{time} + \text{horse} * \text{time} + \epsilon$, where the effect of horse was considered random, and the interaction term was used as the error term for the evaluation of time. A two-sided hypothesis with $\alpha = 0.05$ was used to determine significance of the main effect of time. A statistical software package[1] was used for the analyses. Where there was a significant effect of time, comparisons with baseline (time=0) were made, using adjusted least squares means with a Dunnett's test maintaining an experiment-wise error of $\alpha = 0.05$. Thus, where a difference from baseline was noted, the *P*-value was ≤ 0.05.

2.3 Results

2.3.1 Clinical signs of disease — Mucous membrane color and capillary refill time did not change across time. Respiratory rate (baseline, 12 breaths/min) was significantly increased at the 0.6 mg/kg/min (25.5 breaths/min) and 0.75 to 1.0 mg/kg/min infusion rates, but returned to pre-infusion values on discontinuation of the ATP-$MgCl_2$ infusion. Rectal temperature (baseline = 38.08 °C) decreased significantly during the 0.9 mg/kg/min infusion rate (37.72 °C) and remained decreased throughout the study.

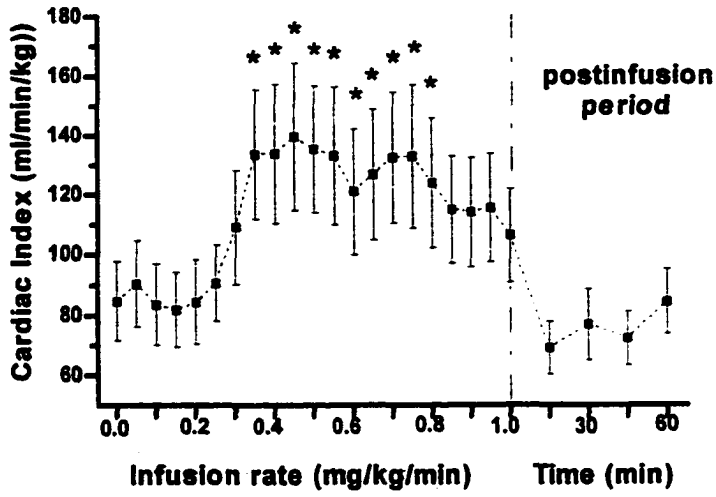
There was a change in gastrointestinal borborygmi activity across time for all quadrants. Intestinal motility decreased, starting at the 0.6 mg/kg/min rate, and were absent from 0.8-1.0 mg/kg/min. Borborygmi were detected but decreased at 15 minutes after infusion and were back to normal by 30 minutes after infusion. Five horses had signs of mild abdominal discomfort at infusion rates > 0.70 mg/kg/min; 2 of these horses became extremely agitated and uncomfortable during the maximal infusion rate.

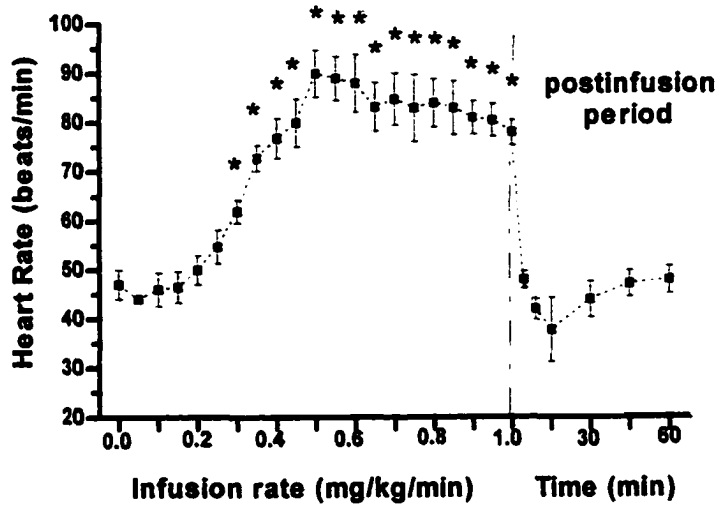
All signs of abdominal discomfort and agitation ceased immediately on discontinuation of the ATP-$MgCl_2$ infusion.

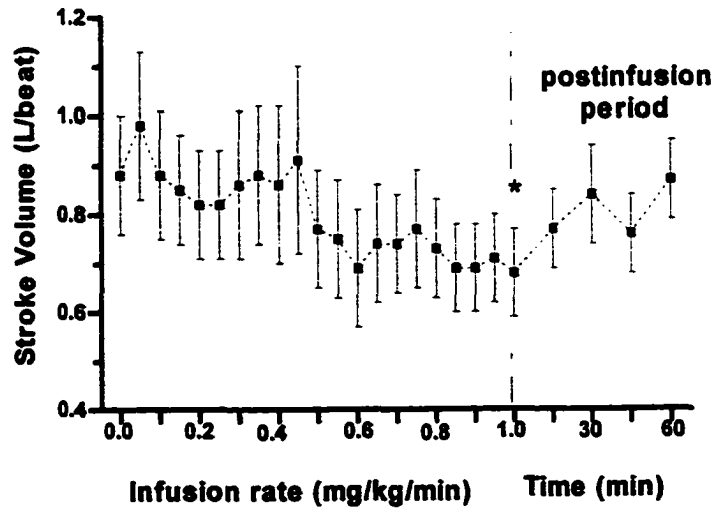
Sweating in the flank region was observed in all horses as the infusion rate increased. Infusion rate at which flank sweating was observed varied among horses; however, lowest infusion rate at which it was observed was 0.45 mg/kg/min. One horse developed muscle fasciculations and profuse, whole-body sweating during the 0.85 to 1.0 mg/kg/min infusion rates. Two horses developed jugular pulses at the higher infusion rates (> 0.75 mg/kg/min). All horses appeared lethargic, and their appetites diminished at infusion rates ≥ 0.40 mg/kg/min. Once infusion was stopped, all horses began to eat, and their overall demeanor improved.

2.3.2 Hemodynamic variables — Cardiac index and HR were significantly increased during infusion (Fig 2.1). Cardiac index returned to preinfusion values at 0.85 mg/kg/min, while HR returned to preinfusion values upon discontinuation of the ATP-$MgCl_2$ infusion. Stroke volume decreased across time, but was significantly decreased only at the 1.0 mg/kg/min infusion rate. Cardiac output was significantly increased between 0.35 and 0.8 mg/kg/min infusion rates. Three horses developed intermittent premature ventricular contractions (PVC) during the study period that seemed to be associated with cardiac instrumentation rather than ATP-$MgCl_2$ infusion, because they were evident prior to the start of the infusion. Systemic vascular resistance and MAP were significantly decreased during the infusion and returned to preinfusion values on discontinuation of ATP-$MgCl_2$ infusion (Fig 2.2). Systolic and diastolic systemic arterial pressures followed a pattern similar to that of MAP. Pulmonary vascular resistance did not change across time; however, MPAP was significantly increased

Figure 2.1 - Mean (±SEM) cardiac index, heart rate, and stroke volume before, during, and after IV infusion of an ATP-$MgCl_2$ solution. *Significant ($P \leq 0.05$) difference from preinfusion values. Notice the difference in the x-axis scale during and after infusion.







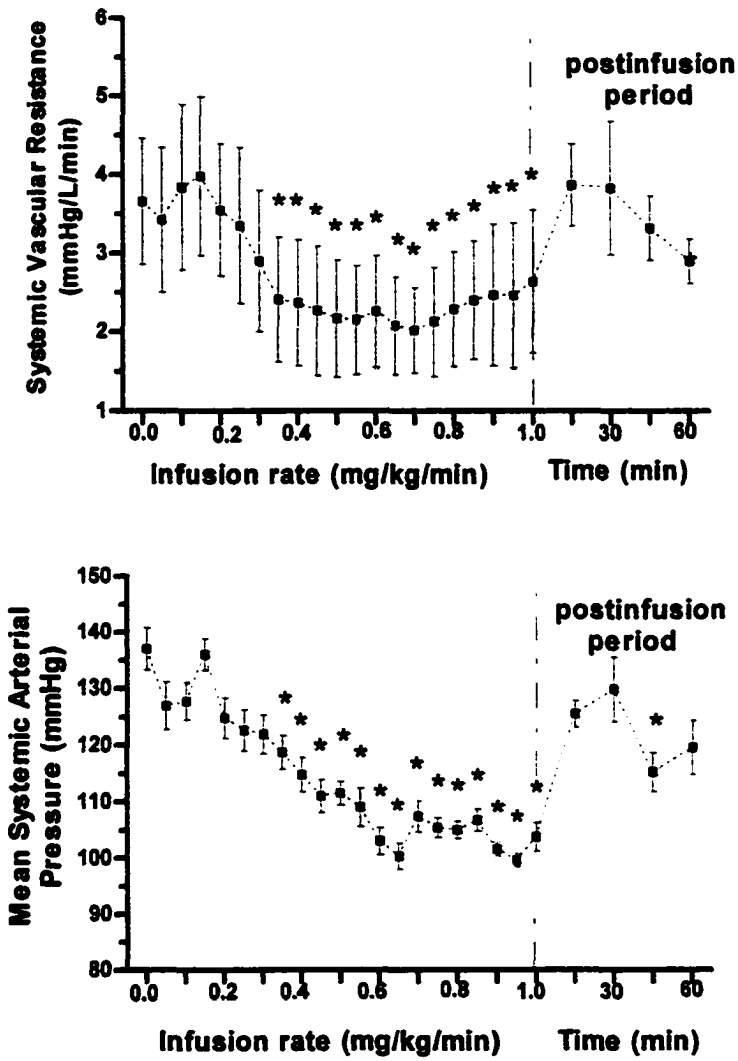


Figure 2.2 - Mean (± SEM) systemic vascular resistance and systemic arterial pressure before, during, and after IV infusion of an ATP-$MgCl_2$ solution. *See* Figure 1 for key.

during the infusion (Fig 2.3). Systolic PAP was significantly increased at the 0.45, 0.55, 0.60, and 0.7 mg/kg/min infusion rates, and DPAP was significantly increased during the 0.35 and 0.45 to 0.80 mg/kg/min infusion rates and at 15 minutes after discontinuation of the infusion.

2.3.3 Metabolic variables — Arterial blood gas values indicated a significant decrease in $PaCO_2$ and HCO_3^- concentrations during the infusion. Once ATP-$MgCl_2$ infusion was stopped, $PaCO_2$ returned to preinfusion values. However, HCO_3^- concentration remained decreased throughout the study. Arterial pH was significantly increased during the 0.75 and 0.8 mg/kg/min infusion rates, then significantly decreased from baseline at all times after discontinuation of infusion (Fig 2.4). The PaO_2 and SaO_2 were significantly increased during the 0.7 and 0.95 mg/kg/min and 0.75 mg/kg/min infusion rates, respectively. The CaO_2 did not significantly change across time; however, DO_2 was significantly increased between 0.35 and 0.8 mg/kg/min infusion rates (Fig 2.5).

2.3.4 Hematologic variables — Packed cell volume was significantly increased during the 1.0 mg/kg/min infusion rate. Plasma total solids concentration was significantly decreased beginning at 0.2 mg/kg/min and remained decreased throughout the study (Fig 2.6). Total WBC count was significantly increased at 2 and 6 hours after discontinuation of infusion, but it returned to preinfusion values by 24 hours. At the 1.0 mg/kg/min infusion rate, eosinophils and lymphocytes were significantly decreased. Lymphocytes remained decreased at 1 hour after discontinuation of the infusion. There were no significant changes across time for segmented or band neutrophils, monocytes, basophils, RBC indices, platelets, or fibrinogen concentration.

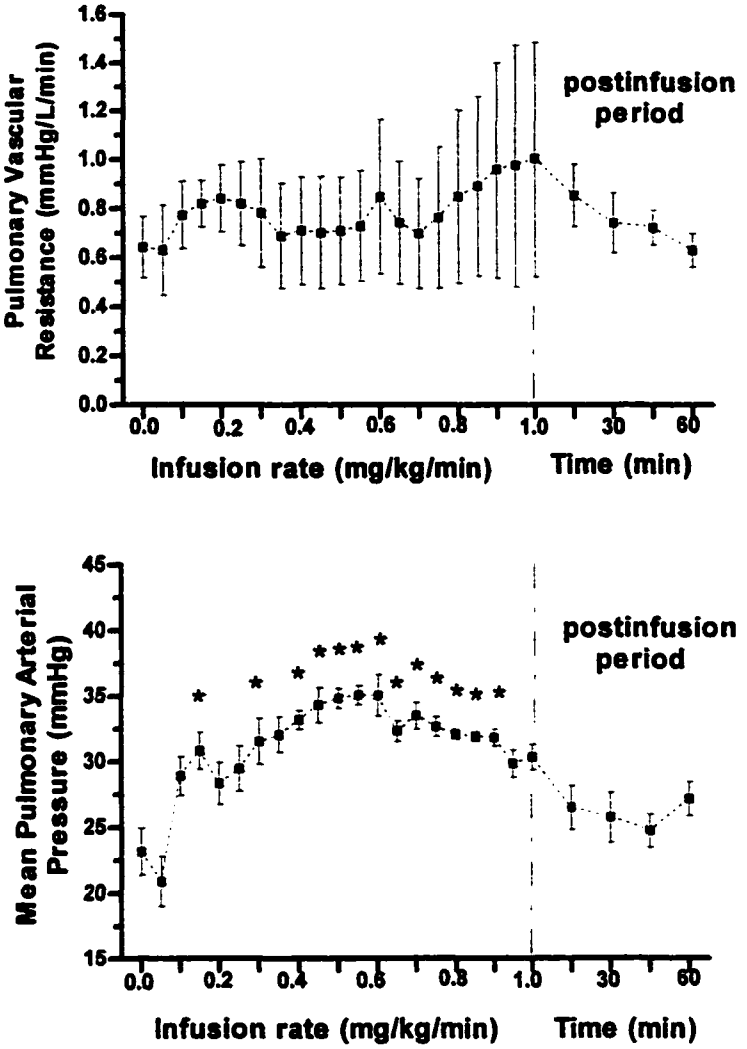
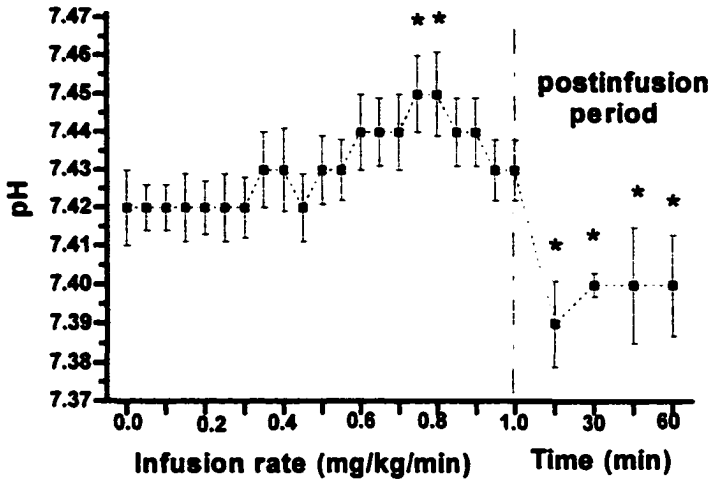
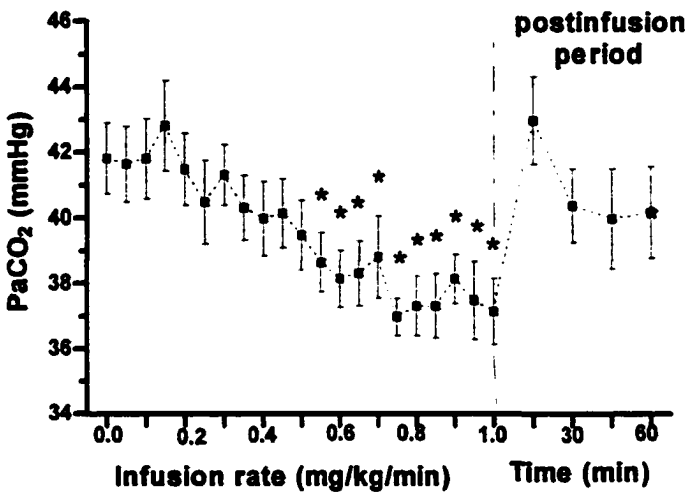
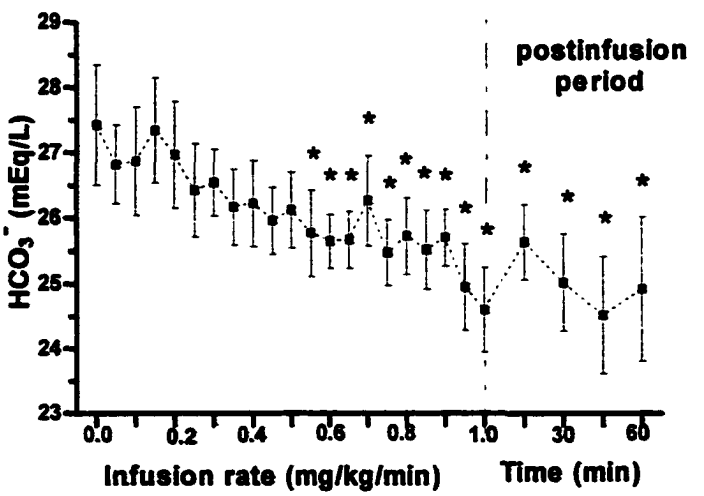


Figure 2.3 - Mean (± SEM) pulmonary vascular resistance and pulmonary arterial pressure before, during and after IV infusion of an ATP-$MgCl_2$ solution. *See* Figure 1 for key.

Figure 2.4 - Mean (± SEM) systemic arterial pH, $PaCO_2$, and HCO_3 concentration before, during, and after IV infusion of an ATP-$MgCl_2$ solution. *See* Figure 1 for key.







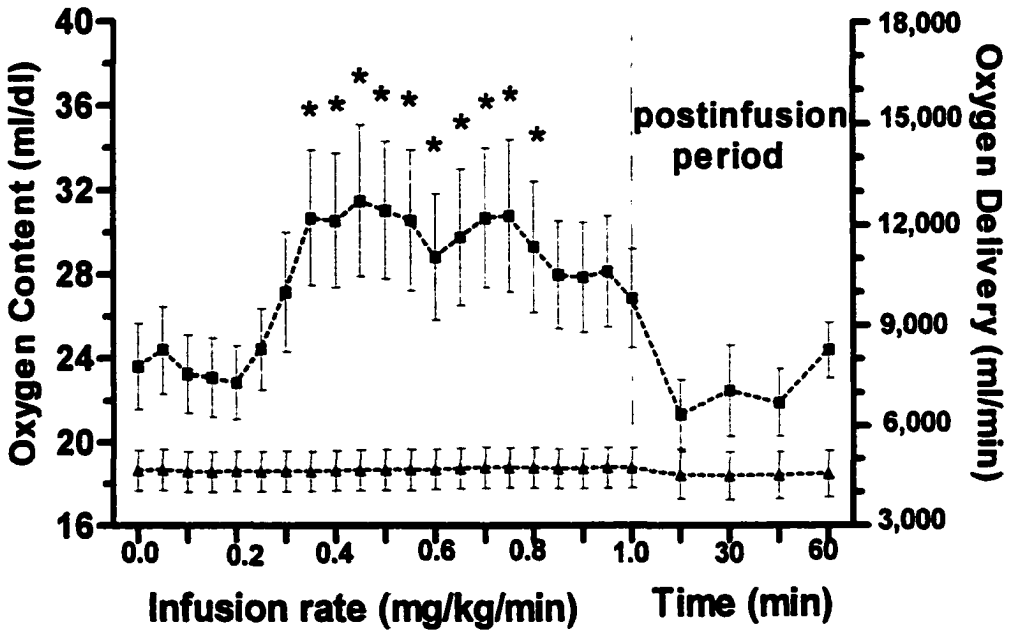


Figure 2.5 - Mean (± SEM) systemic arterial oxygen content (▲) and oxygen delivery (■) before, during, and after IV infusion of an ATP-$MgCl_2$ solution. *See* Figure 1 for key.

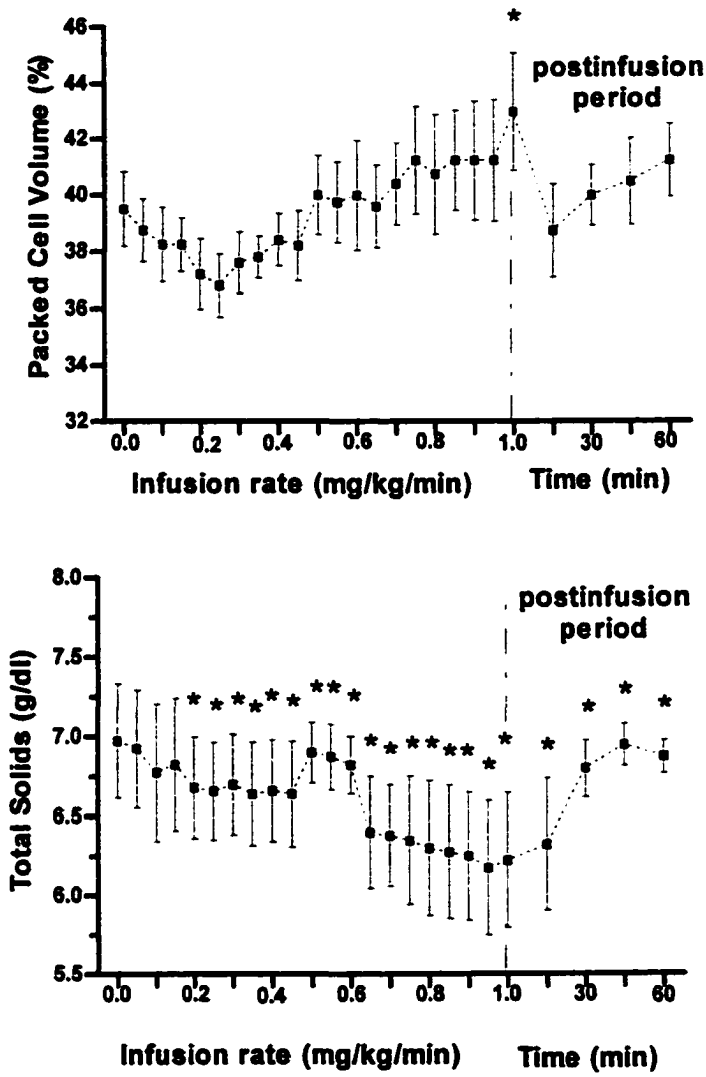


Figure 2.6 - Mean (± SEM) PCV and total solids concentration before, during, and after IV infusion of an ATP-$MgCl_2$ solution. *See* Figure 1 for key.

2.3.5 Serum biochemical variables — There were no significant differences across time for glucose and creatinine concentrations or GGT, ALP or CK activities. Albumin concentration and AST activity were significantly decreased at the 1.0 mg/kg/min infusion rate, and BUN and anion gap were significantly increased at the 1.0 mg/kg/min infusion rate. Bilirubin, globulin, and calcium concentrations were significantly decreased, and phosphorus, sodium, and chloride concentrations were significantly increased at the 1.0 mg/kg/min infusion rate and 60 minutes after discontinuation of infusion. Potassium concentration was significantly decreased at 60 minutes after infusion (Table 2.1).

2.3.6 Urine output — Urine volume was significantly decreased at all infusion rates and returned to preinfusion values once ATP-$MgCl_2$ infusion was discontinued. Urine specific gravity was significantly increased between the 0.3 and 0.85 mg/kg/min infusion rates, then significantly decreased from baseline at 30, 45, and 60 minutes after discontinuation of infusion (Fig 2.7).

2.4 Discussion

Intravenous infusion of a combination of ATP and $MgCl_2$ in clinically normal, conscious, adult horses increased CO, decreased systemic vascular resistance and caused mild pulmonary hypertension. Magnitude of the hemodynamic alterations was dependent on the rate of infusion. For the horses of this study, maximal safe infusion rate was 0.3 mg/kg/min.

Adenosine triphosphate is principally an endothelium-dependent vasodilator that is rapidly metabolized and has a short duration of action (25). It binds to purinoreceptors (P_2) located on vascular smooth muscle (P_{2X} receptor) and endothelial

Table 2.1 - Serum biochemical variables (mean ±SEM) before infusion of an ATP-$MgCl_2$ solution, at a rate of 1.0 mg/kg/min and at 60 minutes after discontinuation of the infusion.

Variable	Pre-Infusion	1.0 mg/kg/min Infusion Rate	Post-infusion (60 min)
Glucose (mg/dL)	113.00±8.50	129.17±10.29	138.17±8.13
Aspartate aminotransferase (U/L)	354.33±91.03	*313.50±81.32	329.00±74.18
Gamma glutamyl transferase (U/L)	12.67±0.92	11.83±0.87	13.67±1.12
Alkaline phosphatase (U/L)	247.67±39.80	271.50±45.01	293.50±59.43
Creatine phosphokinase (U/L)	234.33±34.27	258.67±35.84	280.00±36.82
Bilirubin (mg/dL)	2.28±0.44	*1.93±0.39	*2.02±0.45
Albumin (g/dL)	3.45±0.12	*3.08±0.10	3.30±0.12
Globulin (g/dL)	3.97±0.37	*3.52±0.39	*3.75±0.36
Blood urea nitrogen (mg/dL)	17.17±0.98	*18.67±1.09	18.17±0.95
Creatinine (mg/dL)	1.47±0.11	1.67±0.07	1.53±0.05
Calcium (mg/dL)	12.02±0.24	*10.33±0.08	*11.05±0.21
Phosphorus (mg/dL)	3.12±0.34	*9.53±1.02	*6.73±0.66
Sodium (mmol/L)	135.17±0.83	*137.00±0.52	*137.17±0.31
Potassium (mmol/L)	3.62±0.12	3.68±0.14	*3.10±0.13
Chloride (mmol/L)	99.83±0.83	*101.33±0.76	*101.67±1.15
Anion Gap (mmol/L)	6.60±0.96	*10.33±0.88	7.77±1.46

*Significant ($P \leq 0.05$) difference from preinfusion values.

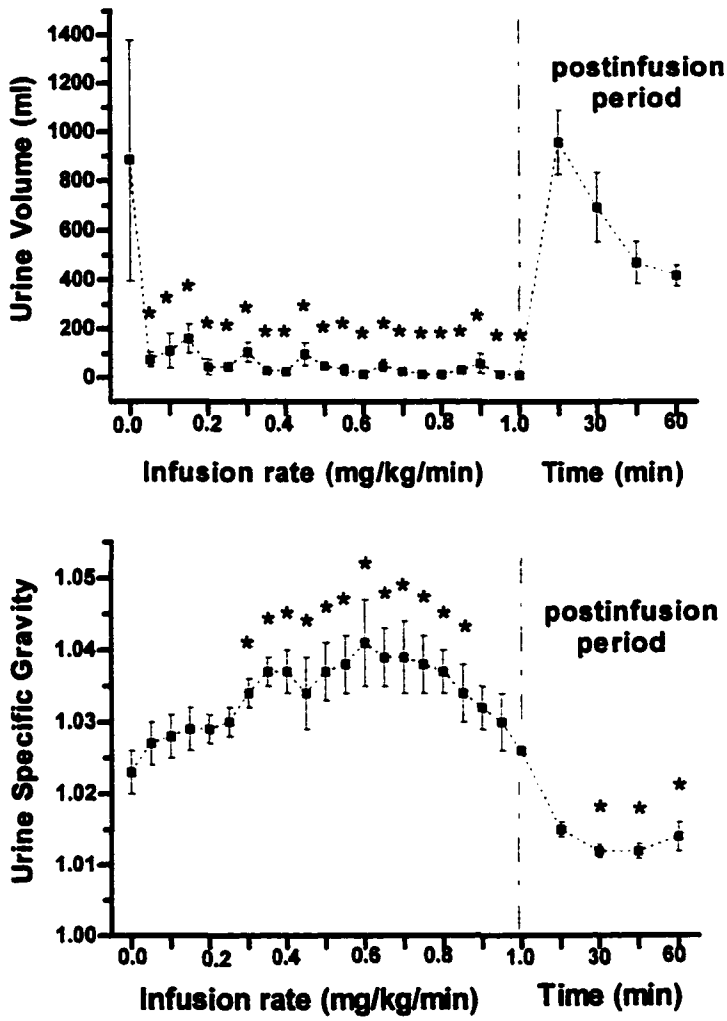


Figure 2.7 - Mean (± SEM) urine volume and specific gravity before, during, and after IV infusion of an ATP-$MgCl_2$ solution. *See* Figure 1 for key.

(P_{2Y} receptor) cells (26). Activation of the excitatory P_{2X} receptor causes vasoconstriction, whereas activation of the inhibitory P_{2Y} receptor causes vasodilatation (26). The vasodilatory effects are attributable to increased formation of nitric oxide, which increases smooth muscle cell concentration of cyclic GMP, the intracellular messenger involved in smooth muscle relaxation (25). When ATP is degraded by ectonucleotidases into adenosine, a P_1-purinoreceptor (A_1-A_3) is activated, leading to vascular smooth muscle relaxation (27). Magnesium also is a potent vasodilator via its important role in regulating arteriolar tone and calcium exchange in vascular smooth muscle (28).

Results of studies have indicated that administration of ATP or $MgCl_2$ alone after shock or ischemia fails to improve organ or animal survival, or both (2). Therefore, it appears that ATP, together with $MgCl_2$ treatment after shock and ischemia, is required for its effectiveness (5). Because ATP and ADP exist in cells as magnesium complexes, and because most ATP reactions require not only ATP as a substrate but also magnesium as a cofactor, administration of magnesium in conjuction with ATP is required for preservation of cell and organ function after a variety of insults (5). Results of other studies have indicated that tissue and mitochondrial magnesium concentrations decrease appreciably after ischemia and reperfusion and that the ATP-$MgCl_2$ combination, but not ATP or $MgCl_2$ alone, substantially increases tissue and mitochondrial magnesium concentrations after ischemia (29).

In clinically normal, conscious, adult males, ATP-$MgCl_2$ infusion (0.2 to 0.4 mg/kg/min) increases CO and HR and causes peripheral vasodilatation without accompanying systemic hypotension (6). Stroke volume also increases by 14%,

suggesting that the ATP-$MgCl_2$ combination may have mild inotropic effects (6). Tachycardia that develops in association with ATP-$MgCl_2$ infusion may be attributed to sympathetic nervous system stimulation (6). Similar increases in CO and HR were observed in our horses at comparable infusion rates. Appreciable changes in HR were not observed during ATP-$MgCl_2$ infusion in rats during shock (11). However, rats were anesthetized, and their baseline HR were higher subsequent to shock. Effects of ATP-$MgCl_2$ administration to horses during hemorrhagic or endotoxic shock or in anesthetized horses may be different than those observed in the study reported here. The hemodynamic effects of IV infusion of ATP-$MgCl_2$ combination during shock in horses warrants further investigation.

As the maximal infusion rate was approached, CO was no longer different from preinfusion values. Stroke volume decreased across time; therefore, despite the sustained increase in HR, CO returned to baseline. The HR may have been too high to allow sufficient atrial and ventricular filling, leading to a decrease in CO (23).

In a study evaluating cardiac catheterization in humans, incidence of complications associated with the procedure was approximately 23% (30). Severity of the complications ranged from mild arrhythmias to acute cardiac perforation (30). Arrhythmias associated with cardiac catheterization procedures in children developed with a frequency of 6.5% (31). Three of 6 horses in the study reported here developed transient PVC without any noticeable deleterious effects.

Infusion of the ATP-$MgCl_2$ combination into the pulmonary artery is a recognized treatment for pulmonary hypertension in children (32). Additionally, pulmonary hypertension secondary to sepsis can be successfully reversed by

administration of the ATP-$MgCl_2$ combination (33). In contrast, horses of this study developed mild pulmonary hypertension during infusion. Despite pulmonary hypertension, pulmonary vascular resistance did not change subsequent to the increase in CO. Results of in vitro studies have indicated that a variation in response to ATP administration can develop in different vascular beds and in vessels under different vascular tensions (26). In certain blood vessels, ATP can stimulate smooth muscle directly (via P_{2X} receptors), causing vasoconstriction (34). In other vascular beds, ATP stimulates the endothelial P_{2Y} receptor, causing vasodilatation (26). If ATP is metabolized to adenosine, activation of the P_1-purinoreceptors can lead to vascular relaxation (27). Under resting tension, ATP induces vasoconstriction in certain vascular beds; however, if tension is increased, vasodilatation occurs (26). Responsiveness of vessels to ATP also differs, depending on the concentration of ATP administered. Low concentrations cause vasodilatation, whereas high concentrations cause transient contraction followed by relaxation (27).

Another explanation for the pulmonary hypertension that developed in our horses is that ATP and norepinephrine can act as cotransmitters from the sympathetic nerves in some tissues (27). This cotransmission enhances norepinephrine- and ATP-induced vasoconstriction. However, ATP-induced vasoconstriction may be masked by its potent relaxant effects mediated by the endothelium (27).

All horses became lethargic, and their appetites diminished as the infusion rate increased. Five of six horses manifested signs of abdominal discomfort. Gastrointestinal borborygmi decreased during higher infusion rates. In humans, transient nausea was observed at infusion rates greater than 0.3 mg/kg/min (6). The exact mechanism for

nausea observed in humans and alterations in behavior and appetite observed in our horses is not known, but could be associated with decreased gastrointestinal motility.

As the rate of the infusion increased, respiratory rate increased and $PaCO_2$ decreased. The increase in respiratory rate likely was attributable to sympathetic nervous system stimulation and pulmonary hypertension. The decreased $PaCO_2$ values and resultant increase in pH likely were associated with tachypnea. Bicarbonate concentration decreased over time. On discontinuation of the infusion, $PaCO_2$ returned to baseline values and HCO_3^- concentration remained decreased, which ultimately resulted in decreased pH. The decrease in HCO_3^- concentration in our horses may have been related to fluid administration during CO determination. Changes in DO_2 paralleled changes in CO, which was expected, because CaO_2 was not significantly altered across time, and because DO_2 is the product of CaO_2 and CO. Similar results were obtained in a canine hemorrhagic shock model in which CaO_2 did not change across time; however, myocardial oxygen delivery was increased because of an increase in coronary arterial blood flow (35).

Although total solids concentration decreased over time, PCV was only significantly different from the preinfusion value during the maximal infusion rate. The decrease in total solids concentration was likely associated with hemodilution secondary to fluid administration associated with CO determination (minimum of 1,650 ml/h). A possible reason why PCV did not change accordingly is because of splenic contraction. This became evident when PCV significantly increased at the 1.0 mg/kg/min infusion rate. The leukocytosis observed at 2 and 6 hours after discontinuation of the infusion

may have been associated with a stress or inflammatory response secondary to drug administration.

Numerous serum biochemical alterations were observed in our horses. High phosphorus and sodium concentrations likely were associated with administration of the ATP disodium salt. Chloride concentration likely increased subsequent to administration of the $MgCl_2$. In healthy, conscious humans, significant electrolyte alterations were not observed during ATP-$MgCl_2$ infusion at the recommended therapeutic dose (6). However, our horses received approximately 2.5-fold the recommended therapeutic dose of the ATP-$MgCl_2$ combination over the course of the study. The decrease in potassium concentration could be secondary to the increased sodium concentration or alkalosis. Albumin and globulin concentrations likely decreased because of hemodilution. Reasons for alterations in AST, bilirubin, calcium, and BUN values observed in our horses are not known. In another study in humans (6), infusion of ATP-$MgCl_2$ did not alter chemistry variables.

The substantial decrease in urine volume after the start of the infusion was related to incorrect determination of baseline urine volume. The urinary bladder should have been emptied several times at 10-minute intervals prior to determining the baseline urine volume, because urine volume was quantified at 10-minute intervals during infusion. The increase in urine specific gravity during infusion may be related to vasoconstriction of the renal vasculature (36). Once infusion was stopped, rebound vasodilatation may have developed, resulting in renal diuresis and a subsequent decrease in urine specific gravity. Results of numerous studies have documented that adenyl compounds have variable and inconsistent effects on the net flow of blood through the

kidney (36). The inconsistency in results suggest that different segments of the renal vasculature may have different responses to adenyl compounds, and responses also vary among species (36).

The effects of volume of ATP-$MgCl_2$ infused during the study may have biased the results obtained, because incremental increases in infusion rate of ATP-$MgCl_2$ are paralleled by increasing volumes of infusion. However, the authors do not believe that the volume of the ATP-$MgCl_2$ preparation infused (maximum of 16.65 ml/horse/min) was sufficient to significantly affect the results obtained in this study.

The maximal safe infusion rate for the horses of the study reported here was 0.3 mg/kg/min. Our criteria for determination of this maximum rate were based on the maximal value prior to which significant alterations in hemodynamic variables were observed. At a rate of 0.35 mg/kg/min, CI, MAP, and SR_L were significantly different from baseline values. Therefore, we selected the 0.3 mg/kg/min rate as our maximal, safe infusion rate.

In conclusion, IV administration of an ATP-$MgCl_2$ combination in healthy, conscious, adult horses resulted in various metabolic and hemodynamic alterations that are without appreciable detrimental effects. Further investigation into the various responses of regional vascular beds and tissues to ATP-$MgCl_2$ administration in horses are necessary before this combination can be recommended clinically as a therapeutic agent during low-flow or septic conditions.

2.5 Product Information

[a]Angiocath, Becton Dickson Infusion Therapy Systems Inc, Sandy, Utah.

[b]Quik-Cath, Baxter Healthcare Corporation, Deerfield, Ill.

[c]Pentalumen thermodilution catheter 41216-01, Abbott Critical Care Systems, Abbott Laboratories, Hospital Products Division, North Chicago, Ill.

[d]Intramedic polyethylene tubing model PE260, Becton Dickson, Sparks, Md.

[e]Normosol, Abbott Laboratories, North Chicago, Ill.

[f]Injector 500, Columbus Instruments, Columbus, Ohio.

[g]Cardio Max II model 85 thermodilution cardiac output computer, Columbus Instruments, Columbus, Ohio.

[h]Polygraph model 7D, Grass Instruments, Quincy, Mass.

[i]Chart recorder model 25-60, Grass Instruments, Quincy, Mass.

[j]Insyte-A arterial catheterization unit model 5820, Becton Dickinson Deseret Medical, Sandy, Utah.

[k]Pressure/ECG monitor model 90602A, Spacelabs Inc, Redmond, Wash.

[l]Uterine flushing catheter model V-PUF-150, Cook Veterinary Products, Bloomington, Ind.

[m]Adenosine 5'-triphosphate disodium salt A3377, Sigma-Aldrich, Inc, St Louis, Mo.

[n]Magnesium chloride hexahydrate M2670, Sigma-Aldrich, Inc, St Louis, Mo.

[o]Cellular acetate filter system 25932-200, Corning, Corning, NY.

[p]Flo-gard 6000 volumetric infusion pump, Travenol Laboratories Inc, Deerfield, Ill.

[q]pH/blood gas analyzer model 238, Chiron Diagnostics Corporation, East Walpole, Mass.

[r]System 9000 automated cell counter, Biochem Immunosystems Inc, Allentown, Pa.

[s]Olympus Reply, Olympus Corporation Clinical Instrument Division, Irving, Tex.

[t]Proc mixed SAS version 6.12, SAS Institute, Cary, NC.

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CHAPTER 3. SYSTEMIC AND LOCAL COLONIC HEMODYNAMIC ALTERATIONS DURING INTRAVENOUS INFUSION OF ATP-$MgCl_2$ IN CLINICALLY HEALTHY ANESTHETIZED HORSES*

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3.1 Introduction

Acute gastrointestinal tract disease (colic) is the leading natural cause of death in adult horses (1,2). Gastrointestinal tract ischemia commonly develops secondary to low-flow/no-flow conditions, with small intestinal volvulus or incarceration (3,4) and large colon volvulus (5-7) being common causes. In one study, strangulating obstructive lesions were associated with the highest mortality (75%) of all types of colic (8). Large colon abnormalities account for up to 50% of the horses that die or are euthanatized subsequent to colic (2-4).

In horses, strangulating volvulus of the ascending colon has been reported to have a mortality approaching 80% (7). The disease is characterized by colonic luminal obstruction and vascular occlusion secondary to the volvulus, thereby resulting in colonic ischemia, mucosal necrosis and vascular thrombosis (9). Colonic blood flow has been shown to remain significantly below baseline values for at least 4 hours after correction of complete arteriovenous occlusion in horses (10). The high mortality associated with colonic volvulus may be related to a sustained reduction of blood flow and hypoperfusion (due to increased vascular resistance) after surgical correction and continued ischemic injury. Endothelial damage occurs in the colonic vasculature subsequent to ischemia-reperfusion and can be exacerbated by endotoxin (11). The sustained decrease in colonic blood flow may be associated with endothelial damage in the colonic circulation, leading to a loss of endothelium-derived vasorelaxants and subsequent vasoconstriction. Many of these horses develop systemic hypotension owing to hypovolemia and endotoxemia, which contribute to decreased splanchnic blood flow. Additionally, colonic mucosal ATP content has been shown to decrease

92% during ischemia and recovers to only 44% of control value after reperfusion, thereby limiting substrate availability for cellular metabolic functions (12). The decreased blood flow and tissue ATP content that occurs during colonic ischemia can lead to disruption of the mucosal barrier and transmural passage of endotoxin into the systemic circulation. If sufficient endotoxin enters the systemic circulation, death can ensue.

Adenosine triphosphate is principally an endothelium-dependent vasodilator that is rapidly metabolized and has a short duration of action (13). The vasodilatory effects of ATP are mediated primarily through activation of purinoreceptors located on endothelial cells (P_{2Y}) (14). The P_{2Y} receptors are coupled to G-proteins and involve second messenger systems (15). Activation of the inhibitory P_{2Y} receptor results in increased formation of nitric oxide (NO), which increases smooth muscle cell concentrations of cyclic GMP, the intracellular messenger involved in smooth muscle relaxation (13). Endothelial-derived hyperpolarizing factor, and possibly prostacyclin, are also generated which contributes to the relaxation response (15). When ATP is degraded by ectonucleotidases into adenosine, an adenosine purinoreceptor (A_2) is activated, leading to vascular smooth muscle relaxation (16). The A_2 receptors are also coupled to G-proteins (15). Magnesium is also a potent vasodilator via its important role in regulating arteriolar tone and calcium exchange in vascular smooth muscle (17).

Administration of adenosine trisphosphate-magnesium chloride combination ($ATP\text{-}MgCl_2$) in humans results in peripheral vasodilatation and increased cardiac output (CO) (18). These findings suggest its potential beneficial use in patients with hypoperfusion (low-flow) or organ ischemia. The use of $ATP\text{-}MgCl_2$ following

hemorrhagic shock and other adverse circulatory conditions in both humans and laboratory animals has been shown to improve mitochondrial function and tissue ATP content (19,20); restore organ function, blood flow, and perfusion (20-23); improve reticuloendothelial function, survival time, and survival rate (24,25); and down regulate the synthesis and release of inflammatory cytokines (26).

We have recently investigated the hemodynamic and metabolic alterations associated with intravenous infusion of ATP-$MgCl_2$ in clinically healthy, conscious, adult horses (27). Intravenous administration of ATP-$MgCl_2$ caused a rate-dependent increase in CO and decrease in systemic vascular resistance (SR_L) without any appreciable detrimental effects. Based on these results, ATP-$MgCl_2$ infusion may potentially increase perfusion to the gastrointestinal tract. Administration of ATP-$MgCl_2$, which has vasodilatory actions, increases CO and delivers an energy substrate (ATP) and co-factor (Mg) directly to the tissues, may offer a potential therapy for horses with intestinal ischemia, endotoxemia and shock. Therefore, the purpose of this study was to characterize the local colonic and systemic hemodynamic alterations associated with intravenous infusion of ATP-$MgCl_2$ in clinically normal, anesthetized horses. We hypothesized that administration of ATP-$MgCl_2$ would cause a rate-dependent decrease in colonic and systemic vascular resistance, principally via vasodilatation.

3.2 Material and Methods

3.2.1 Horses - The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Twelve clinically healthy grade horses (9 females and 3 castrated males), ranging in age from 3 to 13 (median, 7) years and in body weight from 315 to 461 (median, 395) kg, were studied. All horses were

vaccinated for eastern and western encephalitis and tetanus toxoid 3 months prior to the start of the study. Horses were maintained on pasture prior to the study. Food, but not water, was withheld for 12 hours prior to the study to decrease the colon contents, which facilitated its manipulation.

3.2.2 Instrumentation - Horses were sedated with xylazine hydrochloride[a] (0.5 mg/kg, IV) and butorphanol tartrate[b] (0.02 mg/kg, IV). All catheters were placed percutaneously after aseptic preparation of the skin and desensitization by subcutaneous infiltration of lidocaine. A 14-gauge, 13.3-cm Teflon catheter[c] was inserted into the left jugular vein for administration of anesthetic drugs and isotonic polyionic fluids. A balloon-tipped, flow-directed thermodilution catheter[d], which was used for measurement of CO and pulmonary artery pressures (PAP), was inserted into the right jugular vein and advanced until the distal port was positioned in the main pulmonary artery. Polyethylene tubing[e] (1.77 mm OD) was inserted distal to the left jugular catheter and advanced until the tip was positioned in the right ventricle for infusion of ice-cold polyionic fluids[f] for measurement of CO. A 55-ml volume of fluid was infused over 4 seconds into the right ventricle, using a carbon dioxide-driven injector[g], and the CO was derived on the basis of thermodilution (28). The dead space of the injection catheter was 5 ml. Cardiac output and PAP were recorded, using a CO meter[h]. Thermodilution signal curves were recorded for each cardiac output measurement. Polyethylene tubing[i] (1.57 mm OD) was inserted into the right jugular vein proximal to the thermodilution catheter and was advanced until the tip was positioned in the right atrium for determination of mean right atrial pressure (MRAP). All catheter positions were confirmed by the presence of characteristic pressure wave forms.

General anesthesia was induced with guaifenesin[j] (50 mg/kg, IV)and sodium thiopental[k] (4.4 mg/kg, IV). After anesthesia induction, a loading dose of sodium pentobarbital[l] (7.5 mg/kg, IV) was administered and general anesthesia was maintained by a continuous infusion of sodium pentobarbital (5 to 15 mg/kg/h). Horses were mechanically ventilated[m] with 100% oxygen at a rate of 6 to 12 breaths/min to a peak inspiratory pressure of approximately 20 cm of H_20. Arterial blood gas analyses, packed cell volume (%) and total plasma protein concentrations (g/dl) were monitored during the study to assess each horse's metabolic/anesthetic status and to make adjustments in anesthetic management if necessary. Isotonic polyionic fluids were administered at a rate of 5 to 10 ml/kg/h. Arterial blood pressures were measured, using a 20-gauge, 5.1-cm Teflon catheter[n] placed in the facial artery. A 14-gauge, 5.1-cm Teflon catheter[o] was inserted proximally into both the left and right jugular veins for infusion of the ATP-$MgCl_2$[p] combination and for collection of jugular venous blood, respectively.

All horses were positioned in dorsal recumbency and prepared for surgery. After performing a ventral median celiotomy, the ascending colon was exteriorized, placed on a warm water heating pad[q], and instrumented (Fig 3.1). Doppler ultrasound flow probes (3 mm)[r] were placed externally around the right ventral and dorsal colonic arteries, and colonic blood flow was measured continuously and recorded. A 20-gauge, 5.1-cm Teflon catheter was placed in each artery and vein of the ventral and dorsal colon, distal to the flow probes, for determination of ventral and dorsal colonic arterial and venous pressures. A 14-gauge, 5.1-cm Teflon catheter was placed in the ventral colon vein and a 20-gauge, 5.1-cm Teflon catheter was placed in the ventral colon artery, both distal to the pressure catheters, for collection of colonic venous and arterial blood, respectively.

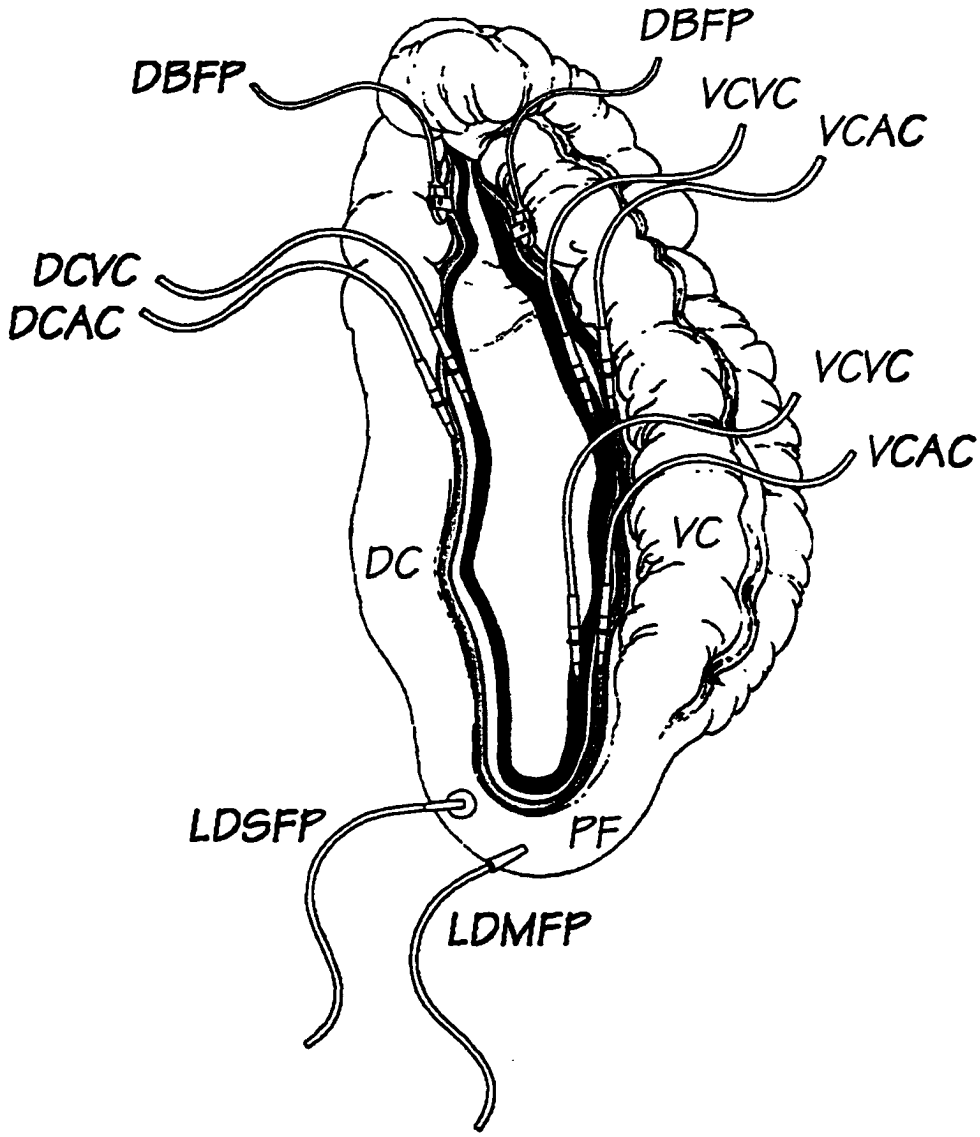

Figure 3.1 - Instrumentation of the equine ascending colon to measure arterial blood flow, arterial and venous pressures, mucosal and serosal perfusion, and collect arterial and venous blood. DBFP = Doppler ultrasound blood flow probes; VCAC = ventral colon arterial catheter; VCVC = ventral colon venous catheter; DCAC = dorsal colon arterial catheter; DCVC = dorsal colon venous catheter; VC = ventral colon; DC = dorsal colon; PF = pelvic flexure; LDSFP = laser Doppler serosal flow probe; LDMFP = laser Doppler mucosal flow probe.

A surface laser Doppler flow probe[s] was positioned on the serosal surface and a needle probe[t] was positioned in the mucosa of the pelvic flexure of the ascending colon to measure seromuscular and mucosal perfusion, respectively. All blood flow probes and pressure transducers were interfaced with physiographs[u], and flow and pressure curves were generated and recorded on chart recorders[v]. A continuous base-apex ECG was also obtained.

3.2.3 Experimental design - The formulation of ATP-$MgCl_2$ has been described (29). Twelve horses were equally and randomly assigned to 1 of 2 groups. Group-1 horses served as saline controls. Group-2 horses received an IV infusion of the ATP-$MgCl_2$ preparation via an infusion pump[w], beginning at a rate of 0.1 mg of ATP/kg of body weight/min. The infusion rate was increased by 0.1 mg/kg/min increments at 10-minute intervals until a maximum rate of 1.0 mg/kg/min was achieved. Unless otherwise stated, data were collected prior to the start of the infusion (time=0), at the end of each infusion rate, at 1-minute intervals for the first 5 minutes after discontinuation of the infusion, and then at 15, 30, 45, and 60 minutes after discontinuation of the infusion. Control horses received an equivalent volume of 0.9% NaCl over the same time. The horses were euthanatized at the conclusion of the study with an overdose of sodium pentobarbital (100 mg/kg, IV).

3.2.4 Systemic hemodynamic variables - Systemic hemodynamic variables that were measured included systolic, diastolic, and mean systemic and pulmonary arterial pressures (SAP, DAP, MAP and SPAP, DPAP, MPAP; mm Hg, respectively), MRAP (mm Hg), and CO (L/min). Three measurements were taken at each time for each pulmonary and facial arterial and right atrial pressure and were used in the analyses.

Five measurements were taken for CO at each time, and the 3 middle values were used in our analyses. Cardiac index (CI; CO ÷ kg of body weight;ml/min/kg), systemic vascular resistance (SR_L; [MAP - MRAP] ÷ CO; mm Hg/L/min), and pulmonary vascular resistance (PR_L; MPAP ÷ CO; mm Hg/L/min) were calculated. Specific ECG alterations were recorded.

3.2.5 Colonic hemodynamic variables - Colonic hemodynamic variables that were measured included mean ventral and dorsal colonic arterial and venous pressures (VCAP, DCAP, VCVP, DCVP; mm Hg, respectively), ventral and dorsal colonic blood flow (VCF and DCF; ml/min), colonic mucosal perfusion (CMP; capillary perfusion units [cpu]), and colonic serosal perfusion (CSP; cpu). Variables that were calculated included overall mean colonic arterial and venous pressures (OCAP; [DCAP + VCAP] ÷ 2 and OCVP; [VCAP - VCVP] ÷ 2; mm Hg), overall mean colonic arterial blood flow (OCF; [VCF + DCF] ÷ 2; ml/min), ventral and dorsal colonic vascular resistances (DCR_L; [DCAP - DCVP] ÷ DCF; mm Hg/ml/min, and VCR_L; [VCAP - VCVP] ÷ VCF; mm Hg/ml/min, respectively), and overall colonic vascular resistance (OCR_L; [DCR_L+VCR_L] ÷2; mm Hg/ml/min).

3.2.6 Nitric oxide analyses - Ventral colonic arterial and venous blood (6 ml each) were collected into tubes containing lithium heparin and processed for immediate analysis of NO concentrations in fresh plasma. Samples were centrifuged at 1,500 X g for 5 minutes and the plasma was harvested and deproteinized by adding 100 µl of trichloroacetic acid (10%) solution to 100 µl of the sample. The samples were vortexed for 30 seconds and then allowed to stand for 15 minutes. The samples were centrifuged at 14,000 x g for 5 minutes. The supernatant was removed for analysis. Aliquots (3 µl)

of plasma were added to a purge chamber of vanadium chloride (100 C) in 1N HCl under a nitrogen atmosphere. Nitric oxide (bound or in the form of nitrate) liberated from the samples into the gaseous headspace was conducted to the NO analyzer[x], where it reacted with ozone to produce a chemiluminescent signal in the 6500-8000 Å range. The amount of light generated was proportional to the NO concentrations, which was calculated from a standard curve of known nitrate concentrations. Each sample was analyzed in triplicate. The limit of detection for the analysis is 1 picomole (1 μM of nitric oxide in 1 μl of plasma).

3.2.7 Statistical analyses - All data were considered continuous and evaluated for normality, using the Shapiro-Wilk statistic. The data were considered to follow a normal distribution if there was failure to reject the null hypothesis of normality at $p \leq 0.05$. Data that were not normally distributed were log transformed, such that they followed a normal distribution. Data were summarized and graphed as mean ± SEM.

All data were analyzed, using the model: $y = \mu + Group + Horse(Group) + Time + Group*Time + Horse*Time + \epsilon$, where y = dependent variable; μ = overall mean; Group = fixed effect of group (0.9% NaCl or ATP); Horse(Group) = random effect of horse nested within Group; Time = fixed effect of time of measurement; Group*Time = fixed effect of Group interaction with Time; Horse*Time = random effect of Horse interaction with Time; and ϵ = residual error.

In this model, the effect of Horse was considered random and was the error term for the evaluation of Group. All other fixed effects were evaluated, using the combined variance of the Horse interaction term and the residual error. A two-sided hypothesis with $p \leq 0.05$ was used to determine significance of the fixed model effects (Group,

Time, Group*Time). Where there were significant model effects, multiple comparisons were made between groups at different time periods, and within groups compared to baseline, using adjusted least squares means, maintaining an experiment-wise error of 0.05. Thus, where a significant difference is noted between time periods and compared to baseline, the p value was ≤ 0.05. Proc univariate and Proc mixed[y] was used for the analyses.

3.3 Results

3.3.1 Systemic hemodynamic variables - There were no significant differences between the control and treatment groups for any measured or calculated variable pre-infusion (baseline). There were no consistently significant changes across time for any measured or calculated systemic hemodynamic variable in group-1 horses with the exception of MRAP (Table 3.1). In group-2 horses, significant changes across time were present for several systemic hemodynamic variables (Table 3.2; Fig 3.2).

3.3.2 Colonic hemodynamic variables - There were no significant differences between the control and treatment groups for any measured or calculated variable pre-infusion. No significant alterations were observed for any local colonic hemodynamic variable across time in group-1 horses (Table 3.3). In group-2 horses, significant decreases in dorsal, ventral, and overall colonic hemodynamic variables were observed across time (Table 3.4, Figs 3.3-3.5). Colonic seromuscular perfusion was significantly decreased across time (Table 3.5).

3.3.3 Nitric oxide analyses - There were no significant differences in colonic arterial or venous plasma NO concentrations across time for group-1 horses. However, significant decreases in colonic arterial and venous plasma NO concentrations were

Table 3.1 - Systemic hemodynamic variables (mean ± SEM) before, during, and after IV infusion of 0.9% NaCl.

Infusion Rate (mg ATP/kg/min)†	CI (mmHg/ml/min)	SAP (mmHg)	DAP (mmHg)	MAP (mmHg)
0.0	43.00±2.00	113.67±5.40	94.72±5.86	100.48±5.76
0.1	42.88±1.83	107.06±5.55	86.06±5.95	93.03±5.78
0.2	41.71±2.13	105.06±4.36	85.50±5.63	92.01±5.16
0.3	43.20±2.44	111.67±4.02	91.50±5.16	98.22±4.74
0.4	40.59±1.74	113.83±5.42	92.56±6.09	99.64±5.79
0.5	45.19±2.40	105.44±5.14	86.28±5.56	92.68±5.36
0.6	43.56±2.52	110.33±6.00	87.72±6.23	95.24±6.14
0.7	42.00±1.96	111.72±5.13	88.11±5.50	95.97±5.37
0.8	44.71±3.28	115.94±5.02	89.83±5.29	98.53±5.16
0.9	44.75±2.43	112.61±5.28	87.89±5.31	96.13±5.27
1.0	43.29±3.08	112.44±4.92	86.94±5.31	95.44±5.15
Time Post-infusion (min)				
1	.	113.50±5.29	89.61±5.23	97.57±5.23
2	.	112.61±5.30	88.78±5.55	96.72±5.45
3	.	112.67±5.37	87.72±5.50	96.06±5.45
5	45.47±2.06	109.78±5.38	86.89±5.41	94.49±5.37
15	42.06±1.78	108.61±5.05	86.28±4.99	93.73±4.98
30	44.60±2.71	106.61±4.43	81.72±4.45	90.01±4.41
45	37.88±2.51	124.42±1.81	100.00±1.55	108.13±1.60
60	41.29±5.00	126.83±1.57	104.17±1.18	111.73±1.25

*Significant ($P \leq 0.05$) difference from pre-infusion values. †Equivalent volume of 0.9% NaCl over the same time period. CI = cardiac index; SAP, DAP, MAP = systolic, diastolic, mean arterial pressure.

Table 3.1 - continued

Infusion Rate (mg ATP/kg/min)†	SPAP (mmHg)	DPAP (mmHg)	MPAP (mmHg)	MRAP (mmHg)
0.0	24.40±4.09	11.97±1.15	17.58±2.18	4.49±0.94
0.1	21.38±2.91	13.15±2.05	16.68±2.37	2.99±1.08*
0.2	23.67±3.31	13.17±2.27	18.58±2.50	3.07±1.08*
0.3	27.35±2.86	16.20±3.31	21.33±2.60	3.46±1.04
0.4	28.17±3.17	16.55±2.45	21.83±2.26	3.38±0.99
0.5	26.38±2.86	15.97±1.22	20.63±1.20	3.71±1.02
0.6	25.80±3.18	16.77±1.63	20.75±1.79	4.20±0.96
0.7	25.63±3.78	13.23±1.32	19.08±1.88	4.01±0.88
0.8	28.47±3.69	14.28±2.24	20.93±1.53	3.66±1.10
0.9	28.20±4.24	13.00±0.89	19.88±1.55	4.56±0.87
1.0	28.10±3.67	13.02±1.47	20.22±0.99	4.61±0.89
Time Post-infusion (min)				
1	.	.	.	4.80±0.86
2	.	.	.	3.90±1.02
3	.	.	.	3.77±0.95
5	25.83±3.16	13.20±1.46	18.93±1.39	3.87±0.84
15	26.57±2.06	16.03±1.89	20.60±1.15	4.24±0.87
30	27.73±1.95	17.67±2.06	22.32±1.46	4.59±0.93
45	30.33±3.84	15.67±5.24	22.67±2.60	4.53±1.23
60	29.58±2.36	15.90±4.15	22.83±2.46	3.42±1.09*

SPAP, DPAP, MPAP = systolic, diastolic, mean pulmonary arterial pressure; MRAP = mean right atrial pressure.

Table 3.2 - Systemic hemodynamic variables (mean ± SEM) before, during, and after IV infusion of ATP-$MgCl_2$.

Infusion Rate (mg ATP/kg/min)	CI (mmHg/ml/min)	SAP (mmHg)	DAP (mmHg)	MAP (mmHg)
0.0	46.61±1.38	120.89±4.28	99.44±3.90	106.60±3.90
0.1	43.50±2.64	117.39±2.88	95.78±2.78	102.99±2.63
0.2	39.78±2.25	115.50±3.91	86.78±2.48*	96.34±2.74*
0.3	41.89±2.30	103.22±5.07*	71.17±3.86*	81.85±4.08*
0.4	45.83±2.02	78.61±4.20*	47.61±3.80*	57.94±3.87*
0.5	48.28±4.49	72.44±4.81*	43.83±4.24*	53.83±4.35*
0.6	52.06±3.61	66.39±5.43*	38.56±4.18*	47.65±4.55*
0.7	53.78±4.09	61.22±5.40*	34.06±2.71*	43.12±3.54*
0.8	53.06±3.84	53.33±5.74*	30.72±2.90*	38.25±3.80*
0.9	44.61±2.32	48.72±5.79*	27.44±2.73*	34.48±3.72*
1.0	44.28±2.61	41.56±5.65*	23.56±2.74*	29.56±3.66*
Time Post-infusion (min)				
1	.	74.94±9.23*	43.39±5.83*	53.91±6.89*
2	.	96.00±7.32*	61.28±5.94*	72.86±6.26*
3	.	96.20±8.26*	62.20±6.95*	73.54±7.24*
5	49.40±3.01	102.47±7.99*	65.80±6.11*	78.03±6.56*
15	46.73±1.80	98.93±6.33*	65.00±4.78*	76.31±5.14*
30	46.93±1.30	105.20±3.58*	74.33±2.40*	84.62±2.65*
45	46.09±1.74	98.67±4.77*	70.50±2.91*	79.88±3.47*
60	43.90±0.97	99.08±1.88*	74.00±1.45*	82.38±1.28*

See Table 3.1 legend for key.

Table 3.2 - continued

Infusion Rate (mg ATP/kg/min)	SPAP (mmHg)	DPAP (mmHg)	MPAP (mmHg)	MRAP (mmHg)
0.0	24.12±3.47	15.80±2.43	19.02±2.73	0.75±1.02
0.1	39.22±8.54*	27.37±6.62*	33.70±8.10*	0.01±1.03
0.2	35.48±4.15*	20.33±0.97	28.32±3.00*	0.55±0.88
0.3	31.22±2.64	19.15±2.64	25.12±1.94	1.30±0.60
0.4	33.83±2.61	20.40±2.77	26.15±1.87	0.94±0.81
0.5	25.73±4.93	20.88±1.56	25.95±1.68	0.31±0.93
0.6	28.95±2.89	18.40±2.46	24.03±2.56	0.46±0.80
0.7	26.38±1.25	17.22±1.57	22.27±1.60	1.56±0.62
0.8	23.83±2.12	16.12±2.30	20.30±2.31	0.93±1.01
0.9	20.63±1.60	12.50±1.91	17.82±2.10	1.57±0.78
1.0	20.95±1.45	13.83±1.49	18.20±1.81	1.33±0.96
Time Post-infusion (min)				
1	.	.	.	0.26±1.07
2	.	.	.	-0.86±0.87*
3	.	.	.	-0.92±1.07*
5	22.34±1.87	16.72±2.16	19.26±1.73	-1.57±0.95*
15	26.20±1.96	17.20±2.54	22.26±1.89	-0.65±0.78
30	24.06±0.42	17.46±0.99	21.66±0.80	-1.82±1.00*
45	24.33±2.33	14.67±1.45	22.00±2.08	-0.18±0.60
60	28.00±4.04	17.33±2.33	22.67±2.60	-1.37±0.89*

See Table 3.1 legend for key.

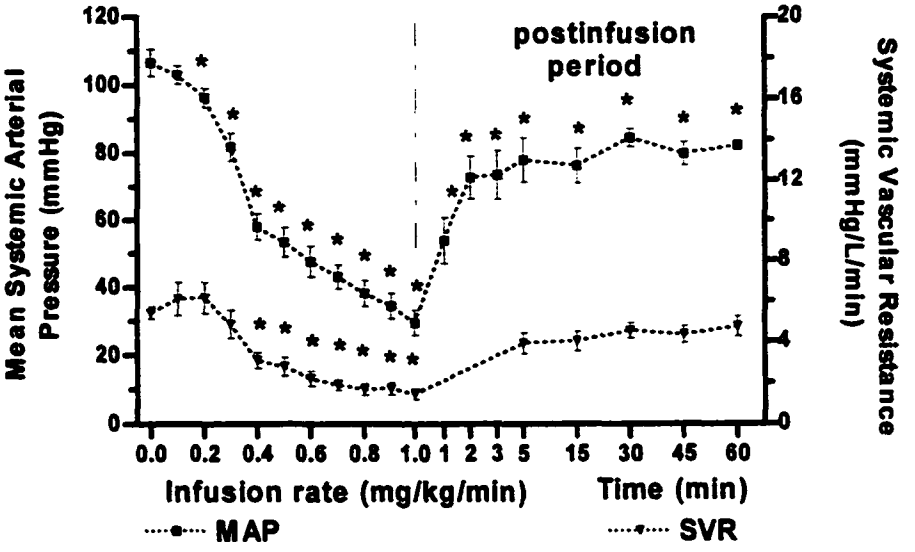

Figure 3.2 - Mean (± SEM) systemic arterial pressure (mmHg) and systemic vascular resistance (mmHg/L/min) before, during, and after IV infusion of ATP-$MgCl_2$ combination. * denotes significant ($P \leq 0.05$) difference from pre-infusion values. Note the difference in the x-axis scale during and after the infusion.

Table 3.3 - Colonic hemodynamic variables (mean ± SEM) before, during, and after IV infusion of 0.9% NaCl.

Infusion Rate (mg ATP/kg/min)†	DCAP (mmHg)	VCAP (mmHg)	DCVP (mmHg)	VCVP (mmHg)
0.0	77.00±6.79	92.67±3.13	9.59±1.32	8.97±1.14
0.1	69.89±6.58	84.33±4.66	9.72±1.15	8.73±1.15
0.2	68.61±7.27	89.00±5.68	9.86±1.23	9.31±1.17
0.3	70.94±5.23	87.56±4.40	9.09±1.07	8.18±1.01
0.4	75.39±6.53	88.06±3.92	9.49±1.17	8.67±1.13
0.5	74.00±6.55	89.50±5.32	9.85±1.21	9.12±1.09
0.6	73.22±7.05	88.33±4.42	9.62±1.18	9.11±1.04
0.7	71.67±7.21	91.11±3.59	9.81±1.13	9.18±1.13
0.8	72.94±6.82	89.06±4.20	10.11±1.11	9.13±1.08
0.9	71.61±7.20	90.83±4.41	9.95±1.09	9.28±1.10
1.0	70.39±7.18	89.28±4.16	10.32±1.16	9.31±1.14
Time Post-infusion (min)				
1	75.93±5.70	90.93±5.62	10.67±1.16	9.49±1.06
2	76.53±6.18	89.20±5.63	10.52±1.16	9.44±1.18
3	70.83±5.78	88.88±4.83	9.86±1.20	9.33±1.14
5	66.00±5.95	88.61±4.68	10.01±1.29	9.28±1.20
15	66.72±5.01	87.28±4.37	10.17±1.35	9.46±1.25
30	70.67±5.32	89.83±4.47	9.94±1.21	9.44±1.12
45	85.67±2.66	94.00±1.99	12.29±1.30	12.88±0.51
60	82.83±3.57	95.75±2.54	12.35±1.19	11.83±0.85

*Significant ($P \leq 0.05$) difference from pre-infusion values. †Equivalent volume of 0.9% NaCl over the same time period. DCAP = dorsal colon arterial pressure; VCAP = ventral colon arterial pressure; DCVP = dorsal colon venous pressure; VCVP = ventral colon venous pressure

Table 3.3 - continued

Infusion Rate (mg ATP/kg/min)†	DCF (ml/min)	VCF (ml/min)	DCR (mmHg/ml/min)	VCR (mmHg/ml/min)
0.0	128.83±6.96	434.56±24.66	0.57±0.14	0.20±0.02
0.1	122.28±7.57	435.78±24.77	0.51±0.11	0.18±0.03
0.2	122.11±6.23	419.83±24.09	0.52±0.13	0.20±0.03
0.3	124.94±8.00	395.83±24.17	0.52 ±0.09	0.22±0.03
0.4	123.61±6.21	433.17±20.34	0.55 ±0.11	0.19±0.02
0.5	113.28±2.00	416.11±18.23	0.56 ±0.09	0.20±0.03
0.6	114.33±4.10	402.17±12.08	0.54 ±0.08	0.20±0.02
0.7	112.72±3.68	430.33±15.41	0.53 ±0.09	0.19±0.02
0.8	131.67±4.37	463.83±23.35	0.49 ±0.09	0.18±0.02
0.9	150.28±13.92	437.17±23.14	0.44 ±0.10	0.20±0.03
1.0	160.39±14.01	463.22±33.41	0.38 ±0.08	0.19±0.03
Time Post-infusion (min)				
1	148.94±15.06	439.67±23.98	0.48±0.06	0.20±0.04
2	162.89±15.79	433.83±25.03	0.44±0.05	0.20±0.04
3	144.00±12.05	420.56±29.22	0.44±0.06	0.21±0.04
5	152.39±13.99	412.94±28.94	0.39 ±0.06	0.21±0.04
15	140.00±11.19	437.61±26.10	0.42 ±0.06	0.19±0.03
30	135.89±11.30	421.00±22.85	0.47 ±0.07	0.20±0.03
45	135.50±3.95	465.58±31.26	0.55 ±0.06	0.18±0.02
60	141.83±6.76	478.75±29.63	0.51 ±0.07	0.18±0.01

DCF = dorsal colon blood flow; VCF = ventral colon blood flow; DCR = dorsal colon resistance; VCR = ventral colon resistance

Table 3.4 - Colonic hemodynamic variables (mean ± SEM) before, during, and after IV infusion of ATP-$MgCl_2$.

Infusion Rate (mg ATP/kg/min)	DCAP (mmHg)	VCAP (mmHg)	DCVP (mmHg)	VCVP (mmHg)
0.0	88.28±2.64	88.56±2.44	9.38±0.93	10.41±1.12
0.1	84.67±2.33	85.72±2.06	8.14±0.95	8.91±1.19*
0.2	79.33±2.50	79.33±2.70	8.43±1.01	9.20±1.25*
0.3	61.61±2.68*	64.83±2.56*	8.22±0.92	8.94±1.06*
0.4	42.78±2.19*	45.22±3.09*	7.87±0.77*	9.00±0.96*
0.5	40.94±3.93*	42.11±3.69*	8.74±0.72	9.66±0.95
0.6	36.11±4.36*	36.89±4.23*	9.42±0.84	9.80±1.05
0.7	35.11±3.72*	36.11±4.49*	8.64±0.81	9.54±0.98
0.8	29.00±2.98*	29.22±3.09*	7.51±0.76*	8.71±1.03*
0.9	23.33±1.45*	26.22±2.64*	7.57±0.80*	8.36±0.95*
1.0	20.70±1.45*	22.28±2.59*	6.59±0.77*	7.54±0.91*
Time Post-infusion (min)				
1	40.78±5.31*	41.72±5.96*	8.36±0.97	9.21±1.09*
2	57.94±5.87*	61.50±5.80*	7.91±0.85*	8.91±0.98*
3	63.27±6.90*	62.40±7.07*	8.39±1.15*	8.75±1.33
5	66.40±5.91*	66.33±6.70*	9.57±1.65	9.65±1.67
15	63.93±4.44*	66.07±4.80*	7.77±1.10*	8.25±1.29*
30	70.27±2.27*	73.60±3.04*	8.36±1.28*	7.81±1.39*
45	69.17±2.49*	68.33±3.51*	9.66±0.68	9.50±0.80*
60	69.08±1.18*	74.08±3.07*	10.67±1.00	10.13±1.10

See Table 3.3 legend for key.

Table 3.4 - Continued

Infusion Rate (mg ATP/kg/min)	DCF (ml/min)	VCF (ml/min)	DCR (mmHg/ml/min)	VCR (mmHg/ml/min)
0.0	129.67±12.72	592.00±54.80	0.73±0.15	0.16±0.03
0.1	139.28 ±14.45	571.67±52.29	0.69±0.16	0.16±0.03
0.2	133.56 ±13.46	514.39±46.27	0.64±0.13	0.16±0.02
0.3	135.61 ±13.42	502.56±70.56	0.43 ±0.05*	0.14±0.02
0.4	115.67±6.86	460.72±41.70*	0.31 ±0.03*	0.09±0.01
0.5	121.33±10.96	431.06±42.24*	0.29 ±0.04*	0.09±0.02
0.6	120.06±12.79	418.00±56.14*	0.23 ±0.04*	0.09±0.02
0.7	129.72±12.07	411.94±59.14*	0.22 ±0.04*	0.10±0.03
0.8	105.33±10.43	374.11±46.17*	0.22 ±0.03*	0.07±0.02*
0.9	93.78±12.16	358.39±46.17*	0.23 ±0.06*	0.06±0.01*
1.0	90.78±12.77*	316.61±41.54*	0.26 ±0.10*	0.09±0.04
Time Post-infusion (min)				
1	133.61±19.65	393.33±34.81*	0.28±0.04*	0.09±0.02
2	118.00±11.23	418.56±39.90*	0.44±0.07*	0.13±0.02
3	132.47±16.00	462.73±57.08*	0.45±0.09*	0.12±0.02
5	142.93±13.39	523.20±55.05	0.42 ±0.07*	0.12±0.02
15	146.40±14.14	529.73±64.80	0.42 ±0.07*	0.12±0.02
30	140.00±12.15	512.07±58.23	0.49 ±0.10	0.15±0.03
45	130.83±13.64	496.75±73.68	0.53 ±0.14	0.14±0.02
60	120.75±11.97	536.67±76.04	0.55 ±0.12	0.14±0.02

See Table 3.3 legend for key.

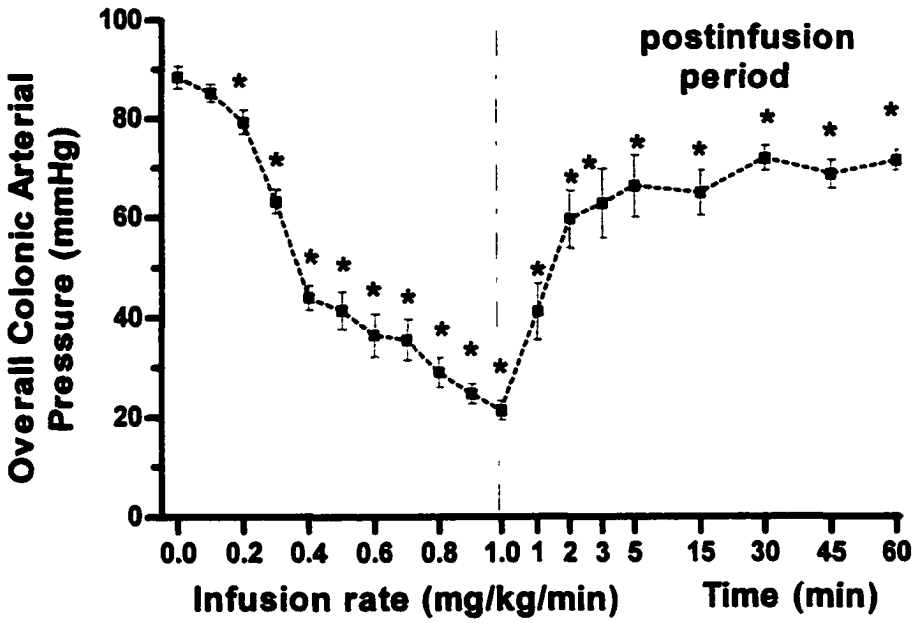

Figure 3.3 - Mean (± SEM) overall colonic arterial pressure (mmHg) before, during, and after IV infusion of ATP-$MgCl_2$ combination. *See* Figure 3.2 legend for key.

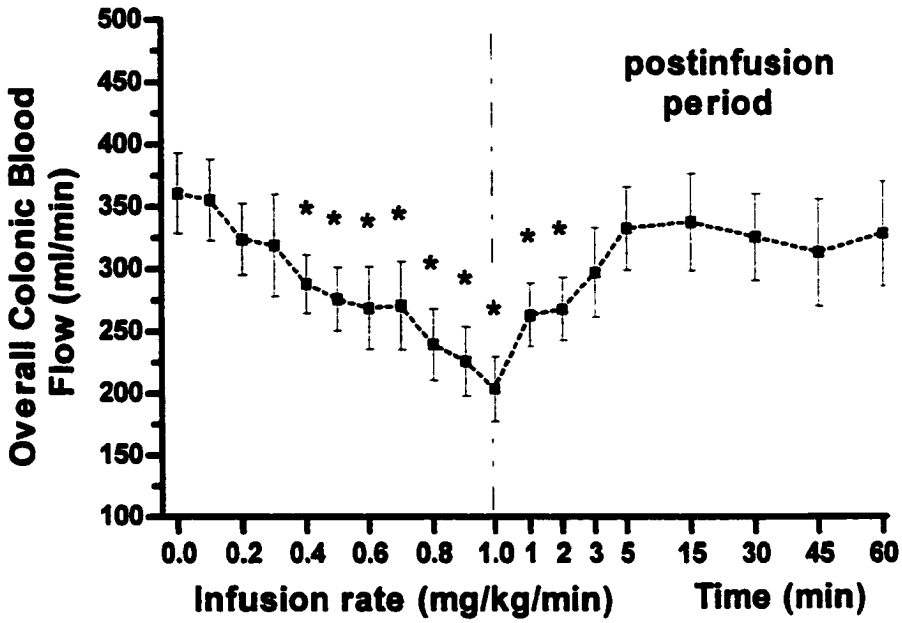

Figure 3.4 - Mean (± SEM) overall colonic arterial blood flow (ml/min) before, during, and after IV infusion of ATP-$MgCl_2$ combination.. *See* Figure 3.2 legend for key.

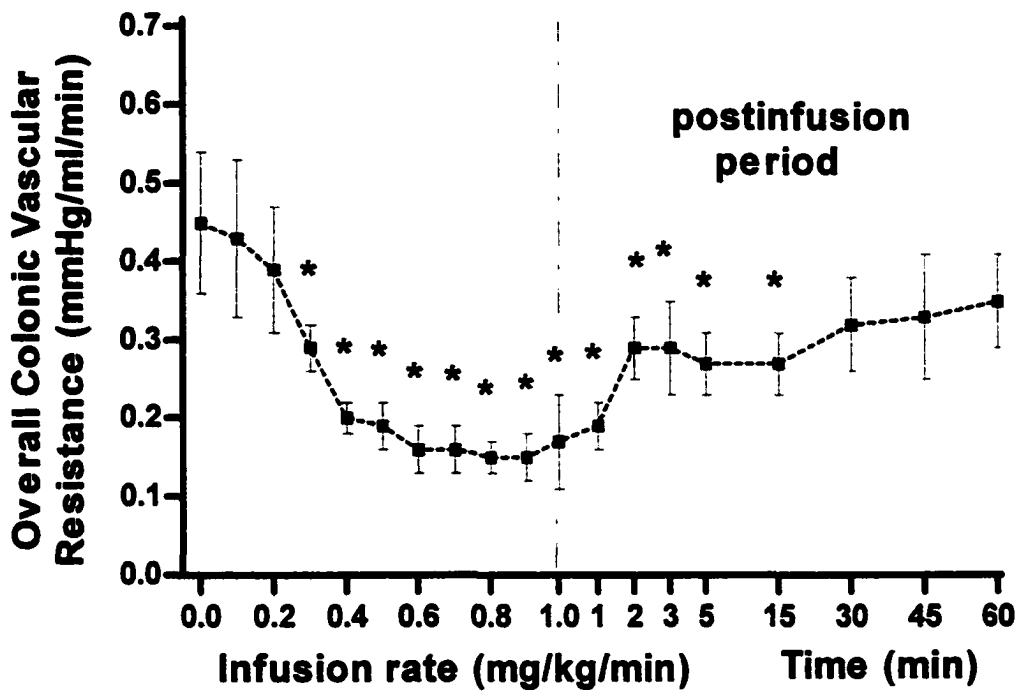

Figure 3.5 - Mean (± SEM) overall colonic vascular resistance (mmHg/ml/min) before, during, and after IV infusion of ATP-$MgCl_2$ combination.. *See* Figure 3.2 legend for key.

Table 3.5 - Colonic mucosal and seromuscular perfusion (mean±SEM) before, during, and after IV infusion of 0.9% NaCl (Group1) or ATP-$MgCl_2$ (Group 2).

	Group 1		Group 2	
Infusion Rate (mg ATP/kg/min)†	**CMP (cpu)**	**CSP (cpu)**	**CMP (cpu)**	**CSP (cpu)**
0.0	14.65±1.61	17.69±2.38	20.51±2.36	25.03±4.09
0.1	13.53±1.26	18.82±2.51	28.41±3.19*	21.36±2.35
0.2	12.06±0.97	18.45±2.00	25.49±3.50	25.30±2.65
0.3	13.01±1.08	16.97±2.14	25.78±2.92	17.72±1.90*
0.4	15.06±1.03	19.56±3.03	24.38±3.25	13.98±0.86*
0.5	12.72±1.16	17.69±3.33	20.36±3.00	16.23±2.57*
0.6	16.79±1.64	18.32±3.08	26.68±5.59	12.24±1.31*
0.7	14.91±1.60	16.25±3.01	22.57±4.86	12.17±1.78*
0.8	16.26±1.37	19.19±3.49	18.01±2.46	10.81±1.59*
0.9	14.69±1.93	20.04±3.60	21.04±4.83	9.68±1.33*
1.0	15.41±1.99	16.73±3.42	18.32±4.08	9.37±1.13*
Time Post-infusion (min)				
1	15.17±1.42	17.92±2.69	22.90±4.52	13.74±1.64*
2	16.56±1.65	18.18±3.04	20.62±2.90	16.52±2.49*
3	15.96±2.05	19.57±3.53	24.11±2.28	21.27±3.44
5	16.04±1.28	18.32±3.11	20.74±2.26	15.60±2.44*
15	18.25±1.60	16.45±2.73	19.71±2.14	16.71±1.69*
30	15.24±1.98	17.61±2.64	29.76±5.57*	14.69±1.37*
45	17.82±1.13	21.64±3.38	15.00±1.17	11.35±2.07*
60	16.20±2.19	18.71±2.67	16.55±2.65	12.07±1.50*

* Significant ($P \leq 0.05$) from preinfusion value

† Equivalent volume of 0.9% NaCl over the same time period for Group 1. CMP = colonic mucosal perfusion; CSP = colonic seromuscular perfusion.

observed in group-2 horses at 45 and 60 minutes after discontinuation of the ATP-$MgCl_2$ infusion.

3.3.4 ECG abnormalities - Six of the 12 horses developed cardiac arrhythmias during the study. Arrhythmias were detected in 1 group-1 horse and 5 group-2 horses. The group-1 horse had atrial premature contractions (APC) prior to the start of the infusion, at the 0.2 and 1.0 mg/kg/min equivalent infusion rates and at 3 minutes post-infusion.

Two group-2 horses developed periods of transient sinus arrest, which were detected in one horse prior to and one horse after the start of the infusion. A third horse had intermittent 2° atrioventricular (AV) block before and after the start of the infusion. A fourth horse had ventricular premature contractions (VPC) prior to the start of the infusion, during the 0.1 to 0.5 mg/kg/min infusion rates and from 0.9 mg/kg/min until the end of the study. At 2 minutes 45 seconds after discontinuation of the infusion, this animal developed ventricular fibrillation and died. The fifth horse had an undefined atrial arrhythmia before and after the start of the infusion.

3.4 Discussion

Intravenous infusion of ATP-$MgCl_2$ in clinically normal, anesthetized adult horses caused a rate-dependent decrease in systemic and colonic vascular resistance, principally via its vasodilatory effects. In contrast to studies performed in other species, mild transient pulmonary hypertension developed in the group-2 horses during the 0.1 and 0.2 mg/kg/min infusion rates. In a previous study, we determined the maximum safe intravenous infusion rate in conscious adult horses to be 0.3 mg ATP/kg/min (27). Administration of ATP-$MgCl_2$ at 0.3 mg/kg/min in the present study caused a

significant decrease in colonic arterial pressure and vascular resistance without a significant decrease in colonic arterial blood flow. However, at rates of 0.4 mg/kg/min or higher, blood flow to the colonic vasculature could not be maintained due to lack of driving pressure secondary to the marked decrease in MAP. Below a critical intravascular pressure, blood flow will not be maintained without increasing driving pressure. The mean MAP pressure during the 0.3 mg/kg/min infusion rate in the previous and present study was 121.9 and 81.85 mm of Hg, respectively (27). The systemic arterial pressure below which insufficient driving pressure is available to maintain blood flow to the splanchnic circulation is not known in horses. In clinical situations, systemic hypotension during general anesthesia is generally not treated until MAP reaches 60 mm of Hg or below. During the infusion rate at which a significant alteration in colonic arterial blood flow was observed (0.4 mg/kg/min), mean MAP was 58 mm of Hg. The results of the present study suggest that an infusion rate of 0.3 mg ATP/kg/min may be beneficial to improve tissue perfusion to the ascending colon following an ischemic insult as long as driving pressure is maintained. However, the rate of infusion of ATP-$MgCl_2$ may need to be lowered in horses with hypotension secondary to hypovolemia or endotoxemia, which occurs commonly with intestinal ischemic diseases.

The systemic and colonic hemodynamic alterations observed in group-2 horses was directly related to administration of ATP-$MgCl_2$. We did not observe significant differences in any measured or calculated variable between the two groups prior to the start of the infusion. Also, there were no consistent significant changes across time in the group-1 horses. Therefore, the hemodynamic alterations that were observed in the

group-2 horses were due to the effects of the ATP-$MgCl_2$ administration and not due to differences between groups, effect of anesthesia or effect of time.

The mechanism of ATP-$MgCl_2$-induced vasodilatation has been investigated. In lambs, administration of N^{ω}-nitro-L-arginine (30) or methylene blue (31) (nitric oxide inhibitor or scavenger) inhibited vasodilatation, whereas indomethacin (prostacyclin inhibitor) and theophylline (32) (adenosine inhibitor) administration did not. Based on these findings, the authors concluded that ATP-$MgCl_2$ exerts its vasodilatory effects through endothelial-derived NO rather than prostacyclin, adenosine or $MgCl_2$ (30,31).

Magnesium has been reported to be a potent vasodilating agent and has been demonstrated to potentiate vasodilatation when combined with ATP in vivo (19). However, we do not believe that magnesium was a major contributor to the vasodilatation response observed in the colonic vasculature in this study. In an in vitro pilot study, we have documented that addition of $MgCl_2$ (10^{-12} to 10^{-4}M) to ATP (equimolar concentrations) did not enhance the relaxation response of equine colonic arterial and venous rings (with intact and denuded endothelium) precontracted with endothelin-1, compared with ATP alone (unpublished data). Additionally, $MgCl_2$ alone did not cause appreciable relaxation of colonic vascular rings (with intact and denuded endothelium) precontracted with endothelin-1. Based on this information, the systemic and colonic vasodilatory responses observed in this study were likely mediated through ATP directly and/or one of its metabolites. However, we do not know whether the in vivo and in vitro vascular response to magnesium are similar. Whether magnesium contributed to the vasodilatory response observed in the systemic circulation in this study is not known.

The magnitude of the hemodynamic alterations produced by intravenous administration of ATP-$MgCl_2$ are dependent on rate of administration, site of infusion, species being studied, whether the subjects are conscious or anesthetized and whether the subjects are clinically healthy or exhibiting signs of systemic disease. In contrast to our conscious horse study where we observed significant increases in CO, CI, HR and PAP across time (27), the group-2 horses in this study did not follow a similar pattern. General anesthesia may override the presumed sympathetic stimulation that accompanies ATP-$MgCl_2$ administration. In healthy, conscious men, rates of 0.1 to 0.4 mg ATP/kg/min resulted in significant increases in CO and HR with no change in MAP (18). A study in healthy, conscious lambs receiving 3 rates of intravenous ATP-$MgCl_2$ (0.1, 0.5, or 1.0 mg/kg/min) revealed no change in HR and MPAP but CO was increased during the highest rate and MAP was decreased at the 0.5 and 1.0 mg/kg/min rates (33). In healthy, anesthetized dogs, rates of 0.6-2.5 mg/kg/min increased CO and decreased MAP (21).

The anesthetized horses in the present study and the conscious horses in our previous study developed pulmonary hypertension, which is in contrast to a study in resting lambs documenting no change in pulmonary artery pressure with ATP-$MgCl_2$ administration (30). The reason for the pulmonary hypertension is not known, however, several possibilities exist. In order for the action of a vasodilating agent to be demonstrated, tissue vasoconstriction may have to occur (14). Results of in vitro studies have indicated that a variation in response to ATP administration can occur in different vascular beds and in vessels under different vascular tensions (14). In certain blood vessels, ATP can stimulate the smooth muscle directly (via P_{2X} receptors),

causing vasoconstriction (34). In other vascular beds, ATP stimulates the endothelial P_{2Y} receptor, causing vasodilatation (14). If ATP is metabolized to adenosine, activation of the A_2 purinoreceptor can lead to vascular relaxation (16). Under resting tension, ATP induces vasoconstriction in some vascular beds; however, if tension is increased, vasodilatation occurs (14).

The data obtained from the laser Doppler flow probes located on the serosal and mucosal surfaces of the ascending colon revealed large variation in recorded values. The most likely explanation for this variation is that the intestine is a continually motile organ. A disadvantage of the technique is its high sensitivity to motion-produced disturbances (35,36). If the laser beam is not directed perpendicular to the tissue, loss of intimate contact of the probe with the tissue occurs resulting in inaccurate values being recorded (35,36). Therefore, the method used in the present study to assess serosal and mucosal blood flow cannot currently be recommended. A superior method to assess blood flow distribution to the different layers of the intestinal tract would be to use either radiolabeled or colored microspheres (37,38).

As previously stated, the mechanism of ATP-induced vasodilatation in other species is through increased production of NO. We did not observe a significant increase in either colonic arterial or venous plasma NO concentrations in our group-2 horses despite significant vasodilatation in the colonic vasculature. Potential reasons for the apparent lack of increase in NO production during ATP-$MgCl_2$ administration include: lack of sensitivity of the assaying method; NO being released on the basalar rather than apical surface of the cell where it could escape more readily into the vascular lumen; ATP-induced vasodilatation occurs via a different mechanism in the horse; or

vasodilatation is a result of the $MgCl_2$ or breakdown products of ATP. Nitric oxide production can occur constitutively or inducibly (39). Adenosine triphosphate-induced vasodilatation occurs through induction of the constitutive form of NO synthase (cNOS) in the endothelium (13). Only picomole quantities of NO are released via cNOS, thereby making small changes in concentrations of NO difficult to detect. The mechanism of action of ATP-induced vasodilatation in isolated equine colonic vascular rings is currently being investigated by our laboratory. The reason for the decrease in NO concentrations observed in the group-2 horses after discontinuation of the infusion is not known but may be due to cessation of stimulation of cNOS.

Short-term control of arterial blood pressure homeostasis is mediated primarily by the baroreceptor reflex (40,41). Acute decreases (increases) in arterial pressure are detected by the arterial baroreceptors and result in reflex increases (decreases) in heart rate (40,41). In the present study, there was failure of the arterial baroreceptor reflex to respond to ATP-$MgCl_2$-induced hypotension. Baroreflex sensitivity is depressed by IV and inhalation-administered anesthetics in numerous species (42-45). Although IV-administered anesthetic-induced depression of the baroreflex has been shown to be less than inhalants (46,47), there was failure of the baroreflex to correct the profound decrease in systemic arterial pressure that occurred in the horses in this study.

Cardiac arrhythmias were observed in 6 of 12 horses in this study. The majority (5 of 6) of arrhythmias occurred in horses administered ATP-$MgCl_2$. However, 4 of 5 horses that received ATP-$MgCl_2$ had evidence of cardiac arrhythmias prior to the start of the infusion. The exact etiology of the arrhythmias in the horses is not known but may be associated with administration of preanesthetic and anesthetic agents and/or

cardiac catheterization. In humans, cardiac catheterization has a reported complication rate of approximately 23% (48). In our previous study, 3 of 6 conscious horses receiving ATP-$MgCl_2$ had cardiac arrhythmias; all of which were present prior to the start of the infusion (27). In those horses, the arrhythmias were suspected to be associated with the cardiac catheters.

The most common cardiac disturbance detected in this group involved slowing of conduction through the heart. Administration of ATP-$MgCl_2$ should be used with caution in horses with pre-existing AV conduction disturbances because both ATP and adenosine are biological compounds with potent depressant activity on the atrioventricular (AV) node (49-51). Because of this effect, ATP has been used to treat supraventricular arrhythmias (52). The antiarrhythmic effects of ATP are produced by blocking the reentry circuit in the AV node (53). Atrioventricular conduction disturbances (1°, 2°, 3° AV block) have been observed during continuous IV infusion of ATP (50). In contrast, ATP has been documented to exert an excitatory effect on intraventricular automaticity (49).

Arrhythmias may be associated with reperfusion of ischemic myocardium and this may be a major progenitor for sudden cardiac death in people (54). The electrophysiological basis for arrhythmias associated with reperfusion appears to be heterogenous electrical recovery, but the precise alterations responsible for malignant versus nonmalignant arrhythmias are unknown (54). The cause of ventricular fibrillation and death in one horse in the current study is not known, but we speculate was attributed to a reperfusion phenomenon of the hypoxic myocardium secondary to the profound systemic hypotension (22.9 mm of Hg MAP during maximal infusion rate) that occurred

during the higher infusion rates. This horse had evidence of premature ventriuclar contractions prior to the start of the infusion, which can be indicative of pre-existing myocardial disease or electrolyte imbalances (55). Electrolyte abnormalities were not detected. Therefore, pre-existing myocardial disease which was exacerbated by profound hypotension and secondary hypoxia may have been the cause of ventricular fibrillation in this horse. Because of the potential for atrioventricular conduction disturbances and the increased potential for arrhythmias to develop secondary to systemic hypotension and myocardial ischemia, heart rate and rhythm should be monitored in horses, especially critically-ill horses, receiving an IV infusion of ATP-$MgCl_2$.

In conclusion, IV administration of ATP-$MgCl_2$ to clinically healthy, anesthetized horses caused a rate-dependent decrease in systemic and colonic vascular resistance. Further studies are required to determine the efficacy of ATP-$MgCl_2$ in the treatment of intestinal ischemia in horses. The reduction in blood flow and decreased mucosal ATP content that persists following correction of experimentally-induced ascending colon ischemia may be attenuated with administration of ATP-$MgCl_2$ by improving blood flow and supplying substrate (ATP) and cofactor (Mg) to the highly metabolically-active mucosal layer.

3.5 Product Information

[a]Rompun, Mobay Corp, Animal Health Division, Shawnee, Kan.

[b]Torbugesic, Fort Dodge Animal Health, Fort Dodge, Iowa.

[c]Angiocath 382269, Becton Dickson Infusion Therapy Systems Inc, Sandy, Utah.

[d]Pentalumen thermodilution catheter 41216-01, Abbott Critical Care Systems, Abbott Laboratories, Hospital Products Division, North Chicago, Ill.

[e]Intramedic polyethylene tubing model PE260, Becton Dickson, Sparks, Md.

[f]Normosol, Abbott Laboratories, North Chicago, Ill.

[g]Injector 500, Columbus Instruments, Columbus, Ohio.

[h]Cardio Max II model 85 thermodilution cardiac output computer, Columbus Instruments, Columbus, Ohio.

[i]Intramedic polyethylene tubing model PE205, Becton Dickson, Sparks, Md.

[j]Guaifenesin 0190-8, Puger Chemical Company Inc, Irvington, NJ.

[k]Pentothal 8912, Abbott Laboratories, North Chicago, Ill.

[l]Sodium pentobarbital injection, The Butler Company, Columbus, Ohio.

[m]Anesthesia Ventilator model NELAC-E, North American Drager, Telford, Pa.

[n]Quick-Cath 2N-11-13, Baxter Healthcare Corporation, Deerfield, Ill.

[o]Quik-Cath 2N-11-10, Baxter Healthcare Corporation, Deerfield, Ill.

[p]Adenosine 5'-triphosphate disodium salt A3377 and magnesium chloride hexahydrate M2670, Sigma-Aldrich Inc, St Louis, Mo.

[q]K module model K-20, Baxter Healthcare Corporation, Pharmaseal Division, Valencia, Ca.

[r]Probe #3S1174 model T206, Transonic Systems Inc, Ithaca, NY.

[s]Probe #HLR1143 model BLF-21D, Transonic Systems Inc, Ithaca, NY.

[t]Probe #HLN1110 model BLF-21D, Transonic Systems Inc, Ithaca, NY.

[u]Polygraph model 7D, Grass Instruments, Quincy, Mass.

[v]Chart recorder model 25-60, Grass Instruments, Quincy, Mass.

[w]Life care pump model 4, Abbott Laboratories, North Chicago, Ill.

[x]Model 280 (NOA), Sievers Instruments Inc, Boulder, Colo.

[y]Proc mixed SAS version 6.12, SAS Institute, Cary, NC.

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CHAPTER 4. EFFECTS OF ATP-$MgCl_2$ COMBINATION ON CLINICAL SIGNS, HEMODYNAMIC, METABOLIC, HEMATOLOGIC, AND SERUM BIOCHEMICAL VARIABLES IN CLINICALLY, HEALTHY, CONSCIOUS HORSES ADMINISTERED LOW-DOSE ENDOTOXIN

4.1 Introduction

Endotoxemia remains the leading cause of death in horses (1,2). Endotoxemia affects all horses regardless of age, breed, gender, or geographic location. The prevalence of endotoxemia in horses with acute gastrointestinal tract disease (colic) admitted to referral veterinary hospitals has been estimated to be approximately 25%, and it represents a major cause of mortality in horses with colic (1,3,4). The most common gastrointestinal tract diseases associated with endotoxemia include enterocolitis/enteritis and intestinal strangulation obstruction, and are often associated with complications such as laminitis and gastrointestinal ileus (5).

There are several inherent mechanisms that restrict transmural movement of endotoxins and bacteria from the gastrointestinal tract lumen, including epithelial cells and intercellular tight junctions, cellular secretions, and lamina propria (2). Disruption of the intestinal mucosal barrier allows transmural passage of endotoxin into the systemic circulation. If sufficient endotoxin enters the portal circulation, the ability of the liver to remove it may be overwhelmed, resulting in systemic endotoxemia (6). In comparison with other species, horses are exquisitely sensitive to the effects of endotoxin (7).

Experimental models of endotoxemia produce clinicopathologic alterations that parallel the changes that occur during the naturally acquired disease (7-9). In an experimental study utilizing an intravenous endotoxin dose of 35 ng/kg, a marked, acute inflammatory response was observed (9). Increases in serum inflammatory cytokines were noted, as well as alterations in clinical signs and hematologic variables, and these changes mimicked the naturally acquired disease (9).

Studies evaluating the efficacy of ATP-$MgCl_2$ combination suggest it has potential beneficial effects in patients with hypoperfusion (low-flow) or organ ischemia. The use of ATP-$MgCl_2$ following hemorrhagic shock and other adverse circulatory conditions in both humans and laboratory animals has been shown to improve mitochondrial function and tissue ATP content (10,11); restore organ function, blood flow, and perfusion (11-14); improve reticuloendothelial function, survival time, and survival rate (15,16); and down-regulate the synthesis and release of inflammatory cytokines (TNF and IL-6) (17).

In our laboratory, IV administration of ATP-$MgCl_2$ to clinically healthy, conscious horses was associated with a rate-dependent increase in cardiac output, decrease in systemic vascular resistance and mild pulmonary hypertension without any appreciable detrimental effects (18). In a second study, IV infusion of ATP-$MgCl_2$ at an infusion rate of 0.3 mg of ATP/kg of body weight/min to clinically healthy, anesthetized horses, caused a significant decrease in colonic vascular resistance without a corresponding decrease in colonic arterial blood flow (19). These results suggest that IV infusion of ATP-$MgCl_2$ could have beneficial effects during low-flow conditions by improving tissue perfusion and providing an energy substrate (ATP) directly to ischemic tissues for maintenance of cellular metabolism.

Administration of ATP-$MgCl_2$, which has vasodilatory actions, increases cardiac output and delivers an energy substrate directly to the tissues, offers a potential therapy for horses with gastrointestinal tract ischemia and endotoxic shock. We hypothesized that IV infusion of ATP-$MgCl_2$ would significantly attenuate the pathophysiologic alterations in clinical signs and cardiopulmonary, metabolic, hematologic, and serum

biochemical variables subsequent to low-dose endotoxin infusion in clinically normal, conscious adult horses. The purpose of the study reported here was to evaluate the effects of IV ATP-$MgCl_2$ on hemodynamic, hematologic, and metabolic alterations during low-dose endotoxin infusion in horses.

4.2 Materials and Methods

4.2.1 Horses - The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Twelve clinically normal horses (8 Thoroughbreds and 4 Quarter Horses; 8 castrated males and 4 females), ranging in age from 6 to 15 (median, 13) years old and weighing 465.9 to 604.5 kg (median, 505.7 kg) were studied. Horses were maintained on a routine preventive health care program. Horses were housed in a box stall and acclimated to the study area (1.82 X 1.82 m) for a minimum of 10 days prior to the start of the study.

4.2.2 Instrumentation - Horses were instrumented, using described techniques (18). All catheters were placed percutaneously after aseptic preparation of the skin and SC infiltration of lidocaine. A 14-gauge, 5.1-cm Teflon-coated catheter[a] was inserted into the left jugular vein for infusion of the pretreatment (LPS or 0.9 % NaCl) and the treatment (0.9% NaCl or ATP-$MgCl_2$) solutions. Polyethylene tubing[b] (outside diameter [OD], 1.57 mm) was inserted distal to the first catheter and advanced until the tip was positioned in the right atrium for determination of mean right atrial pressure (MRAP). A balloon-tipped, flow-directed thermodilution catheter,[c] which was used for measurement of cardiac output (CO) and pulmonary artery pressures (PAP), was inserted distal to the first two catheters and advanced until the distal port was positioned in the pulmonary artery. A 14-gauge, 13.3-cm Teflon-coated catheter[d] was inserted into the right jugular

vein for collection of jugular venous blood. Polyethylene tubing[e] (OD, 1.77 mm) was inserted distal to first catheter and advanced until the tip was positioned in the right ventricle for infusion of ice-cold polyionic fluids[f] for measurement of CO. A 55-ml volume of fluid was infused over 4 seconds into the right ventricle, using a carbon dioxide-driven injector,[g] and the CO was derived on the basis of thermodilution (18). A second polyethylene tubing (OD, 1.77 mm) was placed distal to the first and advanced until the tip was positioned in the pulmonary artery for collection of pulmonary artery blood. Arterial blood pressures were measured by use of a 20-gauge, 4.45-cm Polyurethane-coated catheter[h] placed in the transverse facial artery. All catheter positions were confirmed by presence of characteristic pressure wave forms. All pressure transducers[i] were positioned at the point of the shoulder. The pressure transducers and CO meter[j] were connected to a polygraph[k] and pressure and CO curves recorded on a chart recorder[l]. A continuous base-apex ECG also was obtained.

4.2.3 Experimental design - During the study, horses were cross-tied and hay and water were provided ad libitum. Twelve horses were used in a nested factorial design. Horses were pre-treated with either *E. coli* 055:B5 endotoxin[m] at 35 ng/kg or an equivalent volume of 0.9% NaCl solution via an infusion pump[n] over a 30-minute period. The pre-treatments were randomly assigned. Horses in each pre-treatment group were randomly assigned to one of two treatment groups: 0.9% NaCl or ATP-$MgCl_2$[o]. Immediately upon completion of the pre-treatment infusion (endotoxin or 0.9% NaCl), horses were administered their assigned treatment (100 μmole ATP/kg and 100 μmole $MgCl_2$/kg at an infusion rate of 0.3 mg of ATP/kg of body weight/min or an equivalent volume of 0.9% NaCl solution over the same period). The formulation of

ATP-$MgCl_2$ has been described (18,20). Two weeks later, horses were pre-treated with the opposite solution (endotoxin or 0.9% NaCl) but received the same treatment (ATP-$MgCl_2$ or 0.9% NaCl). The 4 groups were designated: S/S (0.9% NaCl/0.9% NaCl); L/S (LPS/0.9% NaCl); S/A (0.9% NaCl/ATP-$MgCl_2$); and L/A (LPS/ATP-$MgCl_2$). Clinical signs and hemodynamic variables were determined at baseline (BL - prior to the start of the pre-treatment), post-infusion (PI - end of the pre-treatment infusion), 0.05 (5 minutes after starting the treatment infusion) and at 30-minute intervals for 6 hours (hrs) after starting the treatment infusion. Arterial blood gas analyses were determined at BL, PI, 0.5, 1, 1.5, 2, 4, 6, 8, and 12 hrs. Complete blood counts were determined at BL, 0.5, 1, 1.5, 2, 4, 8, 12, 18, and 24 hrs. Serum biochemical profile data were determined at BL, 2, 6, 12, 18, and 24 hrs. All horses were un-instrumented at 6 hrs, with the exception of the transverse facial and pulmonary artery catheters, which were removed at 12 hrs, and one jugular venous catheter, which was removed at 24 hrs.

4.2.4 Clinical signs of disease - Heart rate (beats/min), respiratory rate (breaths/min), rectal temperature (°C), mucous membrane color, capillary refill time (CRT; seconds), and behavior were monitored.

4.2.5 Hemodynamic variables - Hemodynamic variables that were measured included heart rate (beats/min), systolic, diastolic, and mean systemic and pulmonary arterial pressures (SAP, DAP, MAP and SPAP, DPAP, MPAP, respectively; mm Hg), MRAP (mm Hg), and CO (L/min). Three measurements were taken at each time for each pressure variable. Five measurements were taken for CO at each time, and the 3 middle values were used in the analyses. Cardiac index (CI; CO ÷ kg of body weight; ml/min/kg), systemic vascular resistance (SR_L; [MAP-MRAP] ÷ CO; mm Hg/L/min),

and pulmonary vascular resistance (PR_L; MPAP ÷ CO; mm Hg/L/min) were calculated (21). Specific ECG alterations were recorded.

4.2.6 Metabolic variables - Heparinized facial arterial blood samples (2 ml each) were collected anaerobically and stored on ice until analyzed[p] for pH, partial pressure of carbon dioxide ($PaCO_2$; mm Hg), partial pressure of oxygen (PaO_2; mm Hg), percentage oxygen saturation (SaO_2; %), bicarbonate concentration (HCO_3^-; mEq/L), total CO_2 (TCO_2; mmol/L), and base excess. All samples were analyzed within 10 minutes of collection. Systemic arterial oxygen content (CaO_2; ml/dl) was calculated as the sum of oxygen bound to hemoglobin (Hb) and oxygen dissolved in plasma ([Hb x $\%SaO_2$ x 1.34] + [PaO_2 x 0.003]) (18). Oxygen delivery (DO_2; ml/min) was estimated as the product of CaO_2 and CO ($DO_2 = CaO_2$ x CO) (18).

4.2.7 Hematologic variables - Jugular venous blood (3 ml) was collected into tubes containing EDTA. The samples were analyzed for PCV (%), total solids concentration (g/dl), complete blood count[q] and white blood cell differential, and fibrinogen concentration (mg/dl).

4.2.8 Serum biochemical variables - Jugular venous blood (6 ml) was collected into tubes containing lithium heparin and analyzed[r] for plasma glucose (mg/dl), aspartate transaminase (AST; U/L), γ-glutamyltransferase (GGT; U/L), alkaline phosphatase (ALP; U/L),creatine kinase (CK; U/L), total bilirubin (mg/dl), albumin (g/dl), globulin (g/dl), BUN (mg/dl), creatinine (mg/dl), calcium (mg/dl), phosphorus (mg/dl), sodium (mmol/L), potassium (mmol/L), chloride (mmol/L), anion gap (mmol/L), and magnesium (mg/dl).

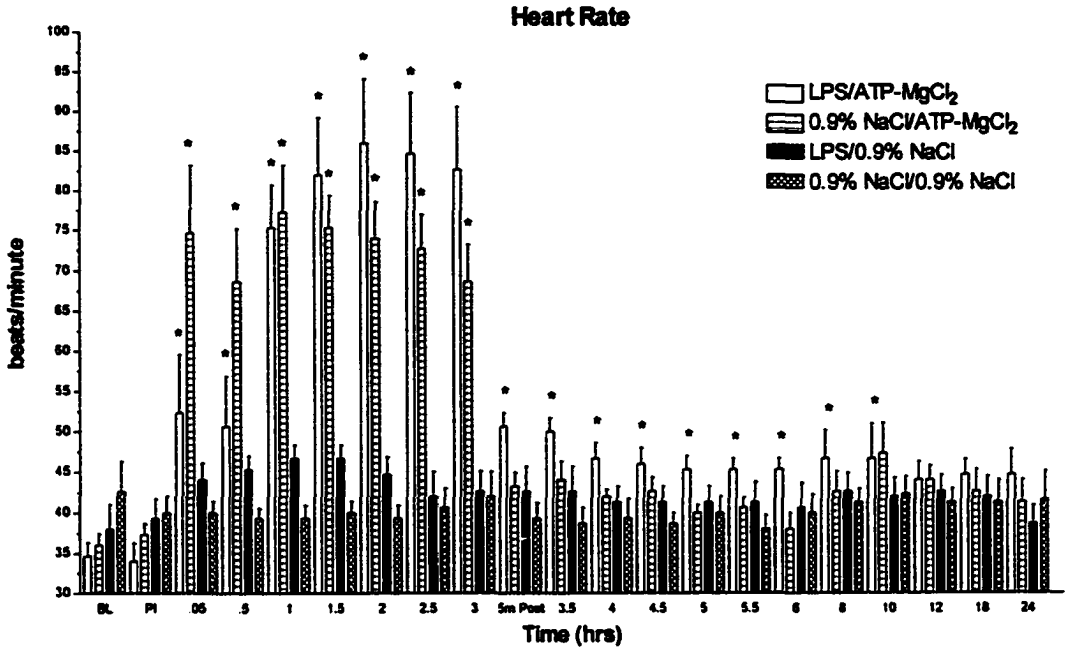
4.2.9 Statistical analyses - The study was considered a mixed effect, nested factorial design, with horses nested within treatments. Data were considered continuous and followed a normal distribution based on the Shapiro-Wilk statistic with failure to reject the null hypothesis of normality at $p<0.05$. Non-normal data was transformed. The data were summarized and graphed as mean ± SEM.

Data were analyzed using a mixed effect general linear model, which accounted for the random effect of horse nested within treatments, and repeated measurements on each horse. Interaction effects were included. Where there were significant effects of time and interactions of treatment, toxin and time at $P<0.05$, pre-determined multiple comparisons were made, using adjusted least squares means maintaining an experiment-wise error of $\alpha = 0.05$. Within group comparisons were made to each groups corresponding BL values to describe behavioral changes over time. Treatments that behaved differently over time were implied to be different. Since S/S and S/A groups functioned as control groups, between group comparisons were made for L/S and L/A groups only when both changed similarly over time from their corresponding BL value. SAS version 8.0 (PROC MIXED, PROC UNIVARIATE)[s] was used for all analyses.

4.3 Results

4.3.1 Clinical signs of disease - Heart rate was significantly increased in S/A and L/A horses (Fig. 4.1). Respiratory rate was significantly increased in S/A and L/A horses (Table 4.1). Rectal temperature was significantly increased in S/A, L/S and L/A horses (Table 4.1). No significant differences in rectal temperature were observed between L/S and L/A horses. There were no consistent changes in mucous membrane

Figure 4.1 - Mean (±SEM) heart rate and cardiac index in horses pretreated with either endotoxin (35 ng/kg over 30 minutes) or an equivalent volume of 0.9% NaCl immediately followed by treatment with either ATP-$MgCl_2$ (100 μmole/kg ATP and 100 μmole/kg $MgCl_2$ at an infusion rate of 0.3 mg ATP/kg/min) or an equivalent volume of 0.9% NaCl. *denotes significant ($p \leq 0.05$) differences from baseline values. Treatment groups that behaved differently over time were implied to be different. Between group comparisons were only made for the LPS/ATP-$MgCl_2$ and LPS/0.9% NaCl groups when both changed similarly over time from their corresponding baseline value. Different letters (a,b) indicate significant differences ($p \leq 0.05$) between L/A and L/S groups.
Key - BL = baseline; PI = end of the pre-treatment infusion; 5m Post = 5 min after discontinuation of the treatment infusion.



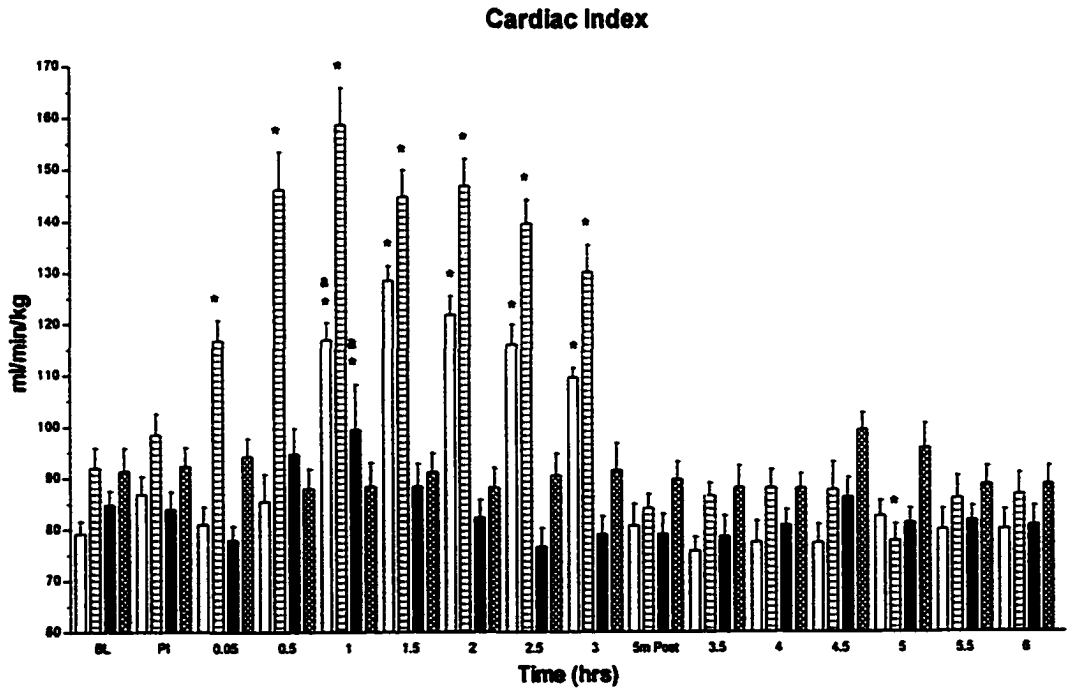


Table 4.1 - Mean (±SEM) respiratory rate and rectal temperature in horses pretreated with either endotoxin (35 ng/kg over 30 minutes) or an equivalent volume of 0.9% NaCl immediately followed by treatment with either ATP-$MgCl_2$ (100 µmole/kg ATP and 100 µmole/kg $MgCl_2$ at an infusion rate of 0.3 mg ATP/kg/min) or an equivalent volume of 0.9% NaCl. *denotes significant ($p \leq 0.05$) differences from baseline values. Key - BL = baseline; PI = end of the pre-treatment infusion; 5m Post = 5 min after discontinuation of the treatment infusion.

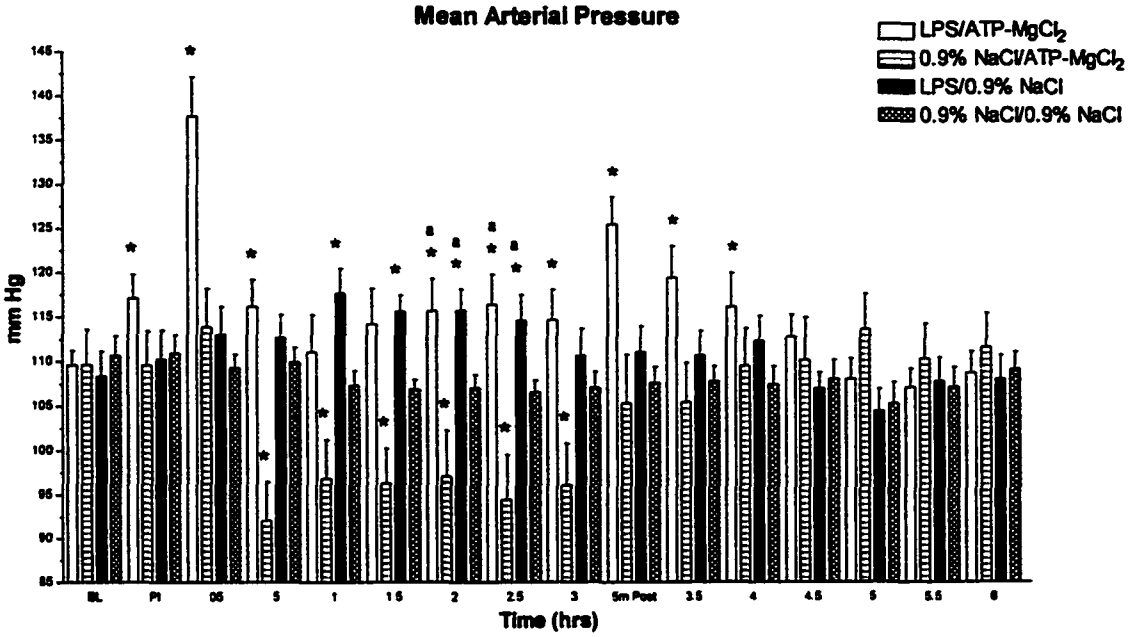
	Respiratory Rate (breaths/min)				Rectal Temperature (°C)			
Time (hrs)	L/A	S/A	L/S	S/S	L/A	S/A	L/S	S/S
BL	13.33	16.00	19.33	19.33	37.75	37.88	37.67	37.93
PI	13.33	16.00	18.00	18.67	37.92	37.78	37.78	37.97
.05	37.33*	27.33*	18.67	15.33	37.93	37.88	37.83	37.88
.5	30.67*	21.33	18.40	17.33	38.15	37.93	37.95	37.95
1	24.00*	20.00	20.00	16.67	38.27*	38.10	38.15*	38.05
1.5	22.67*	20.67	16.67	16.00	38.58*	38.28	38.53*	38.07
2	22.00	24.00	16.00	16.80	39.13*	38.33	39.00*	38.25
2.5	21.33	21.33	18.00	17.33	39.20*	38.28	38.93*	38.35
3	23.33*	21.33	24.00	17.33	39.30*	38.35	39.12*	38.25
5m Post	26.00*	16.00	23.33	17.33	39.08*	38.38*	38.95*	38.27
3.5	25.33*	16.00	25.33	18.00	39.07*	38.32	38.90*	38.32
4	27.67*	16.67	22.00	18.00	38.85*	38.22	38.78*	38.28
4.5	24.67*	18.00	23.33	17.33	38.93*	38.43*	38.60*	38.23
5	24.67*	14.67	19.33	19.33	38.63*	38.43*	38.55*	38.12
5.5	22.67*	16.00	20.00	16.00	38.65*	38.37*	38.48*	38.12
6	22.67*	17.33	20.00	16.67	38.75*	38.47*	38.20*	38.12
8	19.33	20.00	18.00	18.00	38.68*	38.43*	38.33*	38.38
10	18.67	20.67	14.67	17.33	38.55*	38.18	38.18*	38.12
12	16.67	16.67	16.00	16.00	38.37*	38.15	38.07	38.03
18	18.67	19.33	20.00	18.67	37.70	37.83	37.78	37.98
24	19.00	19.67	23.33	18.00	37.57	37.75	37.85	37.82

color and CRT across time for any group. The majority of L/S and L/A horses exhibited signs of transient abdominal pain with subsequent anorexia and depression after endotoxin administration. The behavioral alterations persisted for approximately 2 hrs.

4.3.2 Hemodynamic variables - CO was significantly increased in S/A and L/A horses from 0.05 to 3 and 1 to 3 hrs, respectively, and decreased in S/A horses at 5 hrs. There was a significant increase in L/S horses only at 1 hr. CI followed a similar pattern as CO (Fig. 4.1) MAP significantly increased in L/S and L/A horses and decreased in S/A horses (Fig. 4.2). Systolic and diastolic systemic arterial pressures followed a pattern similar to that of MAP. There was a significant transient increase followed by a decrease in SR_L during the ATP-$MgCl_2$ infusion in L/A horses. Upon discontinuation of the infusion, SR_L transiently increased (Fig 4.2). In S/A horses, SR_L significantly decreased during the ATP-$MgCl_2$ infusion (Fig. 4.2). In L/S horses, there was a significant transient increase in SR_L (Fig. 4.2). In L/S, L/A and S/A horses, MPAP significantly increased (Fig. 4.3). Systolic and diastolic pulmonary arterial pressures followed a pattern similar to MPAP. There was a significant transient increase in PR_L in the L/A, L/S and S/A groups (Fig. 4.3). In L/A and S/A horses, PR_L significantly decreased toward the end of the infusion (Fig. 4.3). There was a significant transient increase in MRAP at 0.05 in both the L/S and L/A groups. Comparisons between L/S and L/A from MRAP could not be performed, because the groups were not equal at BL. In L/A and S/A groups, MRAP was significantly decreased at 0.5 to 2.5 hrs, 5 min post-infusion and 5 to 5.5 hrs and 0.5 hr to 5 min post-infusion, respectively.

4.3.3 Metabolic variables - Arterial pH was significantly increased in L/S horses from PI to 8 hrs, except at 6 hrs. A significant decrease across time for P_{CO_2} was

Figure 4.2 - Mean (±SEM) mean arterial pressure and systemic vascular resistance in horses pretreated with either endotoxin (35 ng/kg over 30 minutes) or an equivalent volume of 0.9% NaCl immediately followed by treatment with either ATP-$MgCl_2$ (100 μmole/kg ATP and 100 μmole/kg $MgCl_2$ at an infusion rate of 0.3 mg ATP/kg/min) or an equivalent volume of 0.9% NaCl. *See* Figure 4.1 legend for key.



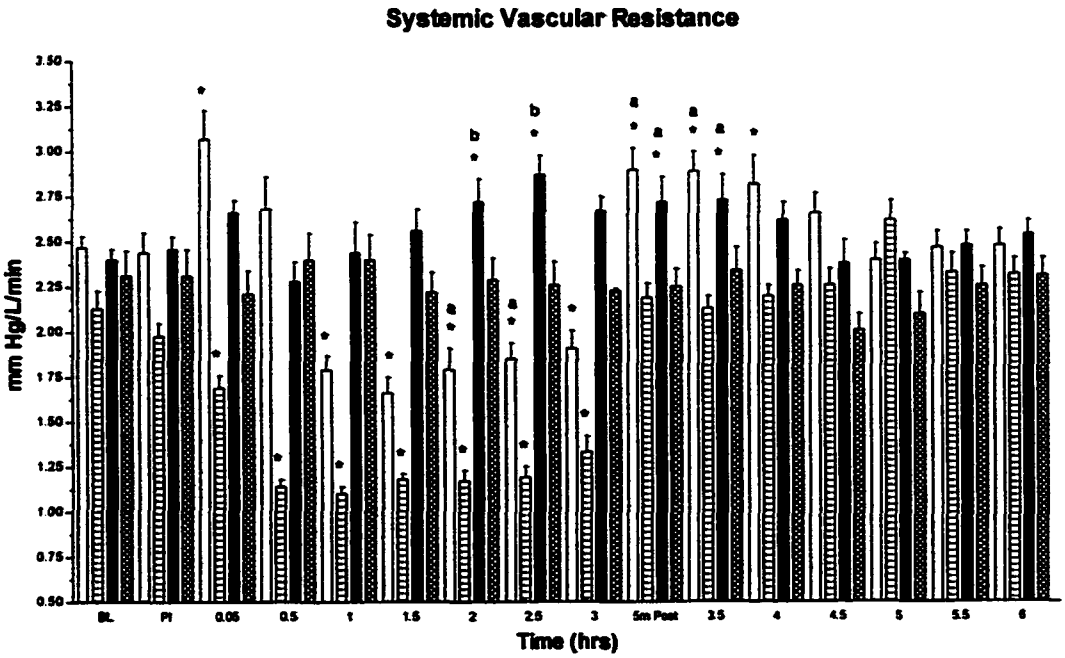
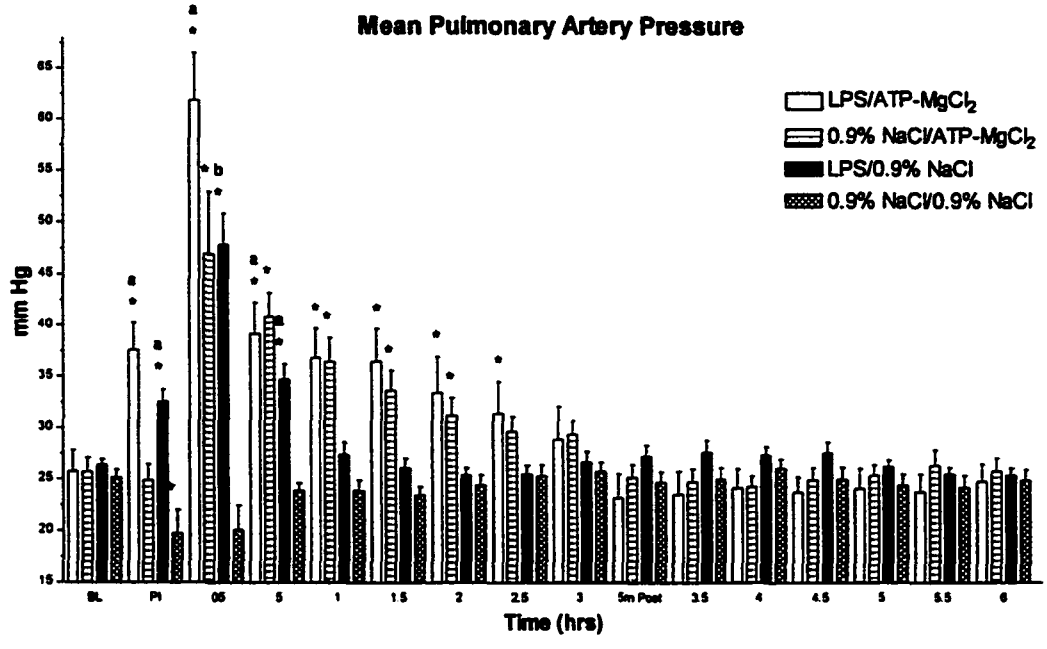
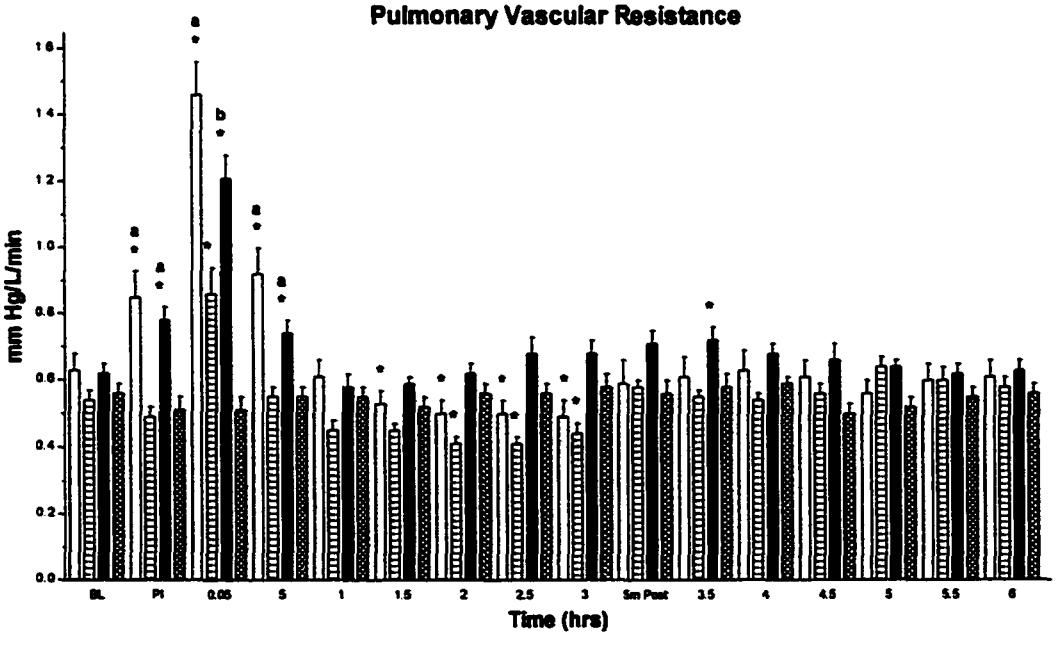


Figure 4.3 - Mean (±SEM) mean pulmonary artery pressure and pulmonary vascular resistance in horses pretreated with either endotoxin (35 ng/kg over 30 minutes) or an equivalent volume of 0.9% NaCl immediately followed by treatment with either ATP-$MgCl_2$ (100 µmole/kg ATP and 100 µmole/kg $MgCl_2$ at an infusion rate of 0.3 mg ATP/kg/min) or an equivalent volume of 0.9% NaCl. *See* Figure 4.1 legend for key.

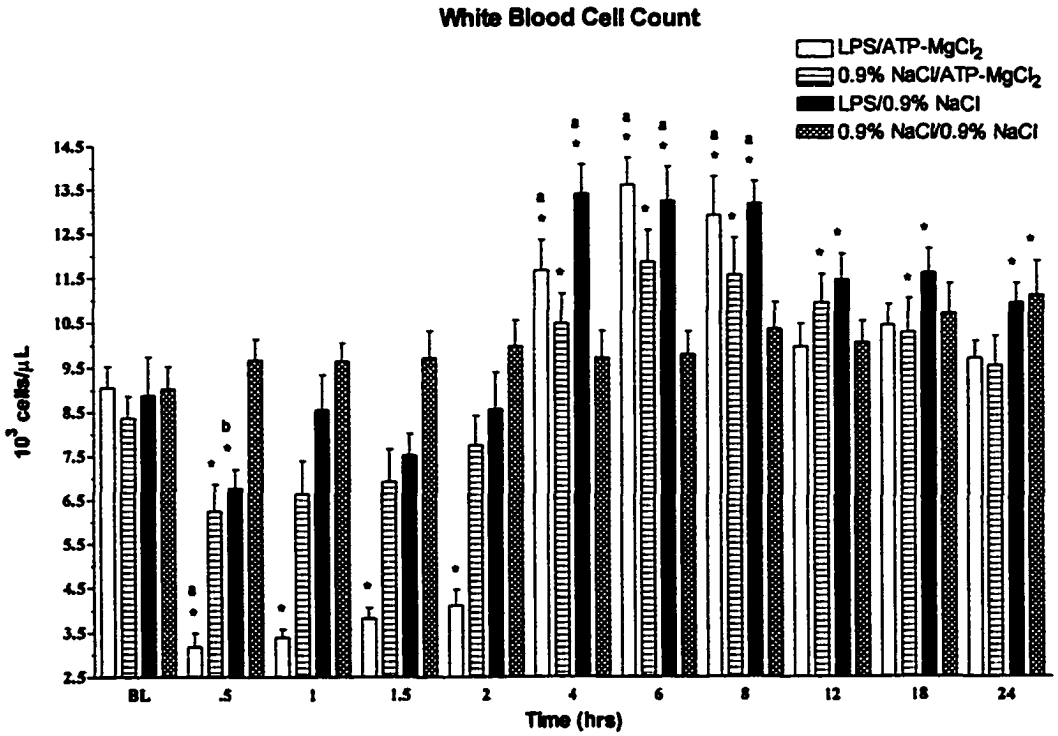


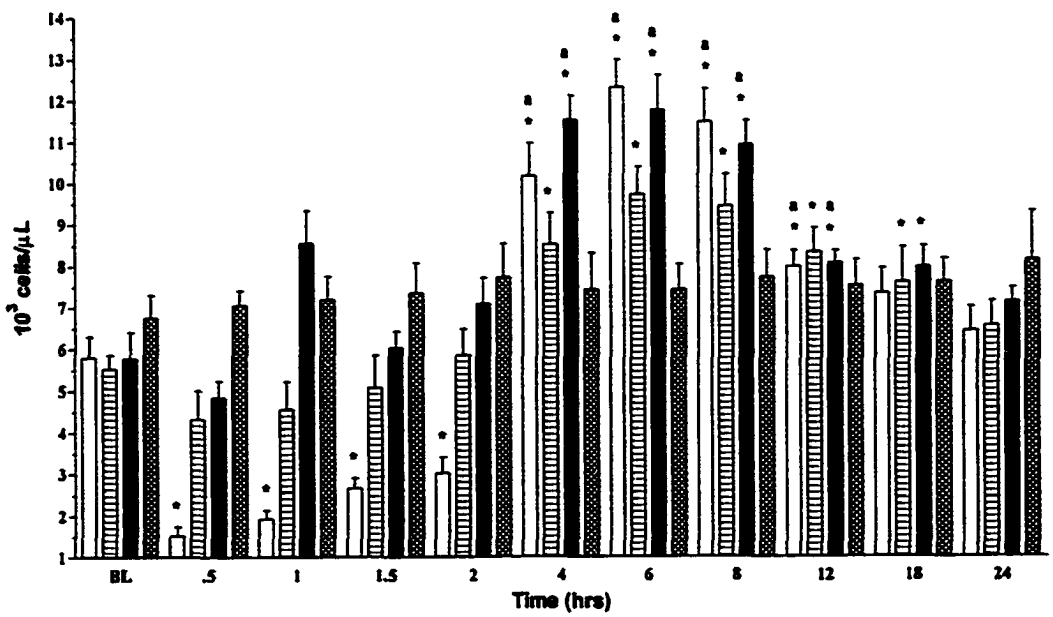


observed in the L/A, L/S and S/S groups. In L/A horses, Po_2 was significantly decreased from 0.5 to 2 hrs. Bicarbonate concentrations and base excess were significantly decreased across time in all groups. TCO_2 was significantly decreased in L/A, L/S, and S/S groups across time. The $\%Sao_2$ was significantly decreased in L/A horses from 0.5 to 2 hrs. There was a significant increase in Cao_2 in L/A and S/A horses at 0.5 and 2 to 6 hrs and at 0.5 to 4 hrs, respectively. Do_2 significantly increased in L/A and S/A horses from 1 to 2 hrs and 0.5 to 2 hrs, respectively.

4.3.4 Hematologic variables - Red blood cell count was significantly increased in L/A, S/A, and S/S horses at 0.5, 2 to 8, and 18 hrs, 0.5 to 4 hrs, and 18 to 24 hrs, respectively. Hemoglobin was significantly increased in L/A and S/a horses at 0.5, 4 to 8, and 18 hrs and 1 to 2 hrs, respectively. Platelets were significantly decreased in the L/A group at 0.5 to 2 and 12 to 24 hrs. There were significant, transient, inconsistent decreases in platelets in the other 3 groups. Fibrinogen concentration was significantly decreased in L/A horses at 4 hrs. White blood cell count was significantly decreased and then increased in L/A, L/S, and S/A horses (Fig. 4.4). Percentage neutrophils were significantly decreased in L/A from 0.5 to 2 hrs and then increased in L/A, L/S, and S/A beginning at 4 hrs (Fig. 4.4). Percentage band neutrophils were significantly increased in L/A at 4 hrs. Percentage lymphocytes were significantly decreased across time in L/A, L/S, and S/A horses. Percentage monocytes were significantly increased in L/S and S/S groups at 6 hrs and at 8 and 12 hrs, respectively. There were no changes in percentage basophils across time for any group. Percentage eosinophils were significantly decreased in L/A and L/S groups at 0.5 to 24 hrs and 6 hrs, respectively.

Figure 4.4 - Mean (±SEM) white blood cell count and neutrophils in horses pretreated with either endotoxin (35 ng/kg over 30 minutes) or an equivalent volume of 0.9% NaCl immediately followed by treatment with either ATP-$MgCl_2$ (100 μmole/kg ATP and 100 μmole/kg $MgCl_2$ at an infusion rate of 0.3 mg ATP/kg/min) or an equivalent volume of 0.9% NaCl. *See* Figure 4.1 legend for key.





4.3.5 Serum biochemical variables - There were no consistently significant changes across time for glucose, AST, and sodium. Numerous mild changes were observed across time in all groups for the remaining serum biochemical variables (Table 4.2).

4.4 Discussion

Intravenous ATP-$MgCl_2$ after low-dose endotoxin administration to clinically healthy, conscious, adult horses failed to attenuate the clinical, hemodynamic, metabolic and hematologic alterations that occur secondary to endotoxin exposure (9,22). The combination of endotoxin and ATP-$MgCl_2$ appeared to potentiate the pulmonary hypertension, leukopenia, and neutropenia that occurred when endotoxin was given alone. Finally, endotoxin and ATP-$MgCl_2$ combination led to thrombocytopenia.

Pulmonary hypertension has been documented to occur in horses given endotoxin (9,23) or ATP-$MgCl_2$ (18,19). The pulmonary hypertensive effects of ATP-$MgCl_2$ and endotoxin in the present study appeared to be additive. However, the pulmonary hypertension and secondary hypoxemia in the L/A group was transient. Furthermore, oxygen delivery was increased in this group owing to an increase in CO. Therefore, the pulmonary hypertension and hypoxemia observed in the L/A horses may not be clinically significant since oxygen delivery was increased.

The development of pulmonary hypertension secondary to ATP-$MgCl_2$ administration appears to be unique in horses. Pulmonary hypertension secondary to cardiac abnormalities in children (24) or in piglets secondary to sepsis (25) can be successfully reversed with administration of ATP-$MgCl_2$. Attenuation of pulmonary hypertension secondary to endotoxin administration was not observed in the present

Table 4.2 - Mean (±SEM) serum biochemical variables in horses pretreated with either endotoxin (35 ng/kg over 30 minutes) or an equivalent volume of 0.9% NaCl immediately followed by treatment with either ATP-$MgCl_2$ (100 µmole/kg ATP and 100 µmole/kg $MgCl_2$ at an infusion rate of 0.3 mg ATP/kg/min) or an equivalent volume of 0.9% NaCl. *denotes significant difference ($p \leq 0.05$) from baseline value. Key - L/A = LPS/ATP-$MgCl_2$; S/A = 0.9% NaCl/ ATP-$MgCl_2$; L/S = LPS/0.9% NaCl; S/S = 0.9% NaCl/0.9% NaCl.

		Time (hours)					
Variable	**Group**	**0**	**2**	**6**	**12**	**18**	**24**
Glucose (mg/dl)	L/A	101.5±6.9	97.7±5.2	109.7±3.8	110±3.4	106.7±2.1	104.8±3.1
	S/A	101±4.5	89.2±5.2	100.5±4.7	100±3.8	101.2±1.3	99.7±3.2
	L/S	96.8±2.5	91.7±4.6	106.5±4.0	101.5±3.8	97.5±2.3	104.7±2.8
	S/S	95.17±2.2	**120.3±6.8***	95.2±2.8	100±2.5	96.3±1.9	107.7±5.0
AST (U/L)	L/A	342±30.7	355.5±30.7	361±41.0	349.3±36.1	331.7±32.2	322.7±34.6
	S/A	277.5±25.8	**305.8±26.5***	297.2±31.9	303.3±30.0	288.17±27.0	287.7±25.9
	L/S	235.2±29.6	233.5±25.6	230±27.6	246.8±31.0	236.67±29.5	234.8±27.1
	S/S	217.5±16.7	222±18.6	223.8±21.0	235.7±18.9	230.7±18.5	223.7±18.7
GGT (U/L)	L/A	10.83±0.7	**23±4.7***	**19.2±4.0***	**19.7±5.6***	**18.5±4.2***	**18±4.9***
	S/A	15±2.6	16.3±2.5	15.3±2.3	15.2±2.2	15.2±1.9	14.3±1.9
	L/S	16.5±5.3	16.7±5.5	17.3±5.4	17.3±5.8	18.5±5.8	17.5±5.7
	S/S	19.5±7.72	19.83±7.71	19.17±7.98	20.5±8.11	21±8.02	19.5±7.5
Alk. Phos. (U/L)	L/A	160.5±9.1	**470.5±75.3***	**316.2±43.6***	**235.7±11.2***	219±10.9	208±11.5
	S/A	150.7±13.3	**223±44.7***	181.3±20.4	170.5±20.4	161.3±17.3	161.8±15.7
	L/S	144±10.1	162.8±11.7	169±11.1	170±11.4	166.3±10.4	166.3±10.4
	S/S	147.7±13.7	151.3±12.0	148.3±11.1	161.5±11.1	160.3±9.9	156.2±8.8
CK (U/L)	L/A	253.3±26.9	279.3±31.8	**297.7±34.6***	276.3±37.5	232.8±31.5	222.8±35.9
	S/A	233.7±37.5	**275.5±29.5***	**271.5±30.6***	257.3±28.0	233.5±27.8	240.7±29.1
	L/S	194.2±20.1	191.5±17.7	177.3±16.9	190.7±21.0	180.2±18.8	181.7±19.0
	S/S	173.8±19.6	184.5±23.0	185±23.9	206.3±27.1	186.7±19.8	174±18.6
Bilirubin (mg/dl)	L/A	2.1±0.4	2.3±0.3	**2.6±0.3***	**3.1±0.3***	**2.7±0.3***	2.25±0.2
	S/A	1.9±0.4	1.8±0.1	1.9±0.2	2.1±0.2	1.9±0.2	1.9±0.2
	L/S	1.4±0.3	1.4±0.2	1.5±0.3	1.6±0.2	1.5±0.2	1.4±0.2
	S/S	1.5±0.2	1.4±0.1	1.4±0.1	1.6±0.2	1.5±0.2	1.5±0.3

Table 4.2 - continued.

Total Protein (g/dl)	L/A	7.4±0.2	7.6±0.2	7.5±0.1	7.5±0.2	7.4±0.2	7.1±0.2
	S/A	6.7±0.2	**7.4±0.2***	**7.2±0.2***	**7.6±0.2***	**7.3±0.1***	**7.3±0.1***
	L/S	7.0±0.2	7.0±0.2	7.0±0.2	**7.5±0.2***	**7.4±0.2***	7.2±0.1
	S/S	6.7±0.2	6.8±0.2	6.8±0.2	**7.3±0.2***	**7.2±0.2***	7.1±0.2
Albumin (g/dl)	L/A	3.3±0.1	3.4±0.0	3.4±0.1	3.4±0.1	3.3±0.1	3.2±0.1
	S/A	3.1±0.1	**3.3±0.1***	3.3±0.1	**3.4±0.0***	3.3±0.0	3.2±0.0
	L/S	3.0±0.1	3±0.1	3±0.1	**3.22±0.1***	3.1±0.1	3.1±0.1
	S/S	2.9±0.1	3.0±0.1	3.0±0.1	**3.17±0.1***	**3.2±0.1***	**3.1±0.1***
Globulin (g/dl)	L/A	4.1±0.2	4.2±0.2	4.2±0.2	4.1±0.2	4.1±0.2	3.9±0.2
	S/A	3.6±0.2	**4.1±0.2***	3.9±0.2	**4.2±0.2***	**4.1±0.1***	**4.1±0.1***
	L/S	4±0.2	4.0±0.2	4±0.2	4.3±0.2	**4.3±0.2***	4.1±0.2
	S/S	3.8±0.3	3.9±0.3	3.9±0.3	4.1±0.3	4.1±0.3	4.0±0.3
BUN (mg/dl)	L/A	17.7±0.8	**20.2±0.8***	**20±0.9***	18.2±1.0	17.7±1.2	17±1.0
	S/A	18±1.2	19.3±2.0	18.5±1.2	18.7±1.2	18.2±1.2	18±1.1
	L/S	14.8±1.1	15.2±1.0	14.7±1.0	13.8±1.0	14.5±0.9	14.8±1.2
	S/S	15.8±1.	16.5±0.8	14.5±1.0	14.3±0.6	15.2±0.9	15.3±0.6
Creatinine (mg/dl)	L/A	1.3±0.1	**1.6±0.1***	1.5±0.1	1.3±0.1	1.2±0.0	**1.2±0.1***
	S/A	1.2±0.1	**1.4±0.1***	1.2±0.1	1.2±0.1	1.1±0.1	1.1±0.1
	L/S	1.3±0.1	1.4±0.1	1.3±0.1	**1.2±0.1***	**1.2±0.1***	**1.2±0.1***
	S/S	1.33±0.11	1.32±0.12	1.3±0.1	1.3±0.1	1.2±0.1	1.2±0.06
Calcium (mg/dl)	L/A	12.0±0.2	11.6±0.2	11.8±0.2	12.1±0.1	12.3±0.1	12.2±0.1
	S/A	11.9±0.2	**12.4±0.2***	12.3±0.2	12.3±0.1	**12.4±0.1***	12.2±0.2
	L/S	12±0.2	11.7±0.2	12±0.4	**12.6±0.1***	**12.5±0.2***	12.4±0.1
	S/S	11.7±0.2	11.9±0.3	**12.2±0.2***	**12.6±0.2***	**12.6±0.2***	**12.3±0.1***
Phosphorus (mg/dl)	L/A	3.6±0.2	**5.7±0.2***	**3.0±0.2***	**2.4±0.1***	**2.0±0.1***	**2.1±0.1***
	S/A	2.9±0.2	**4.9±0.2***	**3.7±0.2***	2.6±0.1	**2.4±0.1***	**2.4±0.1***
	L/S	3.1±0.2	2.9±0.23	**2.12±0.17***	**2.27±0.07***	**1.85±0.09***	**2.15±0.08***
	S/S	3.1±0.3	**2.38±0.20***	**2.42±0.15***	**2.43±0.12***	**2.18±0.13***	**2.05±0.16***

Table 4.2 - continued.

Sodium (mmol/L)	L/A	135.5±0.5	135±0.6	137±0.7	135.7±1.1	135.8±0.7	136.8±0.7
	S/A	135.2±0.8	**137.8±0.8***	136±1.0	136.3±1.2	136±0.8	136.7±0.4
	L/S	135±0.7	135.8±1.1	136.2±0.9	136.5±0.5	135.5±0.4	135.7±0.6
	S/S	135.8±0.8	135.7±0.8	135.8±0.6	136.5±1.0	135.7±0.3	135±0.8
Potassium (mmol/L)	L/A	3.7±0.2	3.6±0.1	**3.2±0.1***	3.7±0.2	**4.3±0.2***	3.7±0.1
	S/A	3.4±0.2	3.7±0.1	3.6±0.2	3.7±0.2	3.6±0.1	3.4±0.1
	L/S	3.4±0.1	3±0.2	3.5±0.1	**4.0±0.1***	**3.9±0.1***	3.7±0.1
	S/S	3.3±0.1	3.5±0.1	**3.8±0.0***	**3.7±0.1***	**3.8±0.1***	3.6±0.1
Chloride (mmol/L)	L/A	100.7±0.7	101.3±0.7	102.8±0.8	102.7±1.2	**103±1.0***	102.5±0.8
	S/A	102±1.1	103.2±0.7	102.3±0.9	101.8±0.9	102.7±0.6	102.2±0.5
	L/S	99±0.9	100.8±0.4	**104±0.9***	**103±0.7***	**102.5±0.4***	**102.5±0.6***
	S/S	99.7±0.5	**102.8±0.6***	**103.3±0.6***	**102.5±0.8***	**102.5±0.9***	101.3±0.9
Anion Gap (mmol/L)	L/A	6.1±0.8	**10.6±0.4***	**8.5±0.2***	7.4±1.0	**8.7±0.8***	7.1±0.9
	S/A	6.5±0.9	**9.5±1.0***	8.0±0.5	8.2±0.6	8.1±0.6	5.8±1.0
	L/S	6.7±0.9	7.3±0.6	6.3±0.7	8.6±1.2	7.8±1.0	6.8±1.1
	S/S	5.3±0.5	6.2±1.0	6.1±0.8	**7.6±0.6***	7.4±1.0	6.1±1.0
Magnesium (mg/dl)	L/A	1.8±0.1	**2.3±0.1***	1.9±0.1	1.7±0.1	1.8±0.0	1.8±0.1
	S/A	1.7±0.1	**2.6±0.1***	**1.9±0.1***	1.7±0.1	1.7±0.1	1.8±0.1
	L/S	1.7±0.1	1.6±0.0	1.8±0.1	1.8±0.1	1.8±0.1	1.8±0.0
	S/S	1.6±0.1	**1.8±0.1***	**1.8±0.1***	**1.9±0.1***	**1.8±0.1***	1.8±0.1

study. The mechanism involved in ATP-$MgCl_2$-induced pulmonary hypertension in horses is not known but may be due to differences in purinergic receptor density in the pulmonary vasculature. ATP can cause vasoconstriction or vasodilatation, principally by activation of purinergic P2X or P2Y receptors, respectively (26). Unlike P2Y receptors which are located on vascular endothelium and require generation of second messengers, P2X receptors are located on vascular smooth muscle cells and use ligand-gated ion channels to induce a response (26). Therefore, response time is faster with activation of P2X receptors. There may be a greater density of P2X receptors in the equine pulmonary vasculature compared with other species. Additionally, when ATP-$MgCl_2$ is administered into the external jugular vein, the pulmonary vasculature is the first major vascular bed encountered. Therefore, it will be exposed to the highest concentration of ATP. When ATP circulates through the lung, the majority of ATP is degraded to other adenine nucleotides and nucleosides by ectonucleotidases (27). Adenosine, a breakdown product of ATP, can activate adenosine/P1 receptors (principally A_2) located on vascular smooth muscle cells leading to vasodilatation. Vascular beds downstream from the lungs will be exposed to higher concentrations of ATP degradation products than ATP itself, thereby causing systemic vasodilatation, which was observed in this study.

Horses administered endotoxin, irrespective of treatment, developed systemic hypertension, whereas horses administered ATP-$MgCl_2$ alone developed systemic hypotension. Intravenous administration of ATP-$MgCl_2$ has been documented to cause a rate-dependent decrease in MAP and SR_L, principally via vasodilatation (18,19). The decrease in SR_L despite the presence of systemic hypertension in L/A horses was

secondary to an increase in CO, which was not observed in L/S horses. The reduction in SR_L observed in L/A horses could lead to improved peripheral perfusion.

White blood cell count decreased in horses administered endotoxin, and the magnitude of the decrease was more pronounced in the group receiving ATP-$MgCl_2$. Neutropenia was only documented in the L/A group. Horses receiving ATP-$MgCl_2$ or LPS alone developed a mild, transient leukopenia without a corresponding neutropenia. The development of leukopenia in S/A horses and the difference in the leukocyte/neutrophil response between L/A and L/S horses may be due to upregulation of adhesion molecules by ATP. Adenine nucleotides have been documented to act directly on leukocytes to enhance expression of the CD11b/CD18 adhesion molecule (28). Additionally, ATP has been demonstrated to stimulate leukocyte adherence to cultured endothelial cells via P2Y and P2U receptor-mediated events (29).

One potential explanation for the apparent negative effects of ATP-$MgCl_2$ on pulmonary artery pressure and leukocyte/neutrophil counts in the L/A group, compared with the L/S group, may be that the response to endotoxin in the L/S group was not as marked. In contrast to a previous study of endotoxin administration in horses (9), the L/S horses in this study did not develop tachycardia, hypoxemia, or neutropenia. Another possible explanation would be that endotoxin was present in our ATP-$MgCl_2$ preparation. When the ATP-$MgCl_2$ solution was analyzed using the Limulus amoebocyte lysate assay, an endotoxin dose equivalent to 1 pg/kg of body weight was detected. Although speculative, we do not believe this dose would cause alterations in clinical signs or hemodynamic and clinicopathologic variables. However, there are no reports in the literature of this dose of endotoxin being administered to horses, so

definitive conclusions regarding the contribution of the endotoxin in our ATP-$MgCl_2$ solution to the overall changes observed in the L/A horses cannot be made. Based on the results of this study, we cannot be certain whether ATP-$MgCl_2$ truly potentiated the negative effects produced by endotoxin administration.

In the L/A, but not L/S, horses developed thrombocytopenia during the ATP-$MgCl_2$ infusion. Endotoxin has been demonstrated to induce platelet aggregation in horses (30). ADP is an agonist of platelets and is present in platelet dense granules along with ATP (31,32). Following exposure to endotoxin, ADP is released from platelet dense granules, contributing to platelet aggregation (33,34). The combination of endotoxin and ATP-$MgCl_2$, some of which will circulate as ADP, most likely contributed to the development of thrombocytopenia, via platelet aggregation, observed in the L/A group.

In conclusion, ATP-$MgCl_2$ administration was not protective during experimentally-induced endotoxemia in clinically, healthy, conscious adult horses. Furthermore, the use of ATP-$MgCl_2$ during endotoxemia may worsen the hemodynamic, metabolic and hematologic status of affected horses. However, the pulmonary hypertension and hypoxemia that developed in the L/A group was transient and accompanied by an increase in oxygen delivery. Since ATP and other adenine nucleotides are released from cells during shock, the potential role of adenine nucleotides in the development of hemodynamic derangements, leukocyte adherence, and coagulopathies during endotoxemia warrants further investigation.

4.5 Product Information

[a]Quik-Cath 2N-11-10, Baxter Healthcare Corporation, Deerfield, Ill.

[b]Intramedic polyethylene tubing model PE205, Becton Dickson, Sparks, Md.

[c]Pentalumen thermodilution catheter 41216-01, Abbott Critical Care Systems, Abbott Laboratories, Hospital Products Division, North Chicago, Ill.

[d]Angiocath 382269, Becton Dickson Infusion Therapy Systems Inc, Sandy, Utah.

[e]Intramedic polyethylene tubing model PE260, Becton Dickson, Sparks, Md.

[f]Normosol, Abbott Laboratories, North Chicago, Ill.

[g]Injector 500, Columbus Instruments, Columbus, Ohio.

[h]Arrow radial artery catheterization set RA-04020, Arrow International Inc, Reading, Pa.

[i]DTX plus DT-6012, Becton Dickinson Infusion Therapy Systems Inc, Sandy, Utah.

[j]Cardio Max II model 85 thermodilution cardiac output computer, Columbus Instruments, Columbus, Ohio.

[k]Polygraph model 7D, Grass Instruments, Quincy, Mass.

[l]Chart recorder model 25-60, Grass Instruments, Quincy, Mass.

[m]Lipopolysacccharide L2880, Sigma-Aldrich Inc, St. Louis, Mo.

[n]Life care pump model 4, Abbott Laboratories, North Chicago, Ill.

[o]Adenosine 5'-triphosphate disodium salt A3377 and magnesium chloride hexahydrate M2670, Sigma-Aldrich Inc, St. Louis, Mo.

[p]pH/blood gas analyzer model 238, Bayer Corp, Norwood, Mass.

[q]Baker systems 9110 plus, Biochem Immunosystems Inc, Allentown, Pa.

[r]AU 600, Olympus Corporation Clinical Instrument Division, Irving, Tex.

[s]SAS statistical software, version 8.0, SAS Institute, Cary, NC.

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CHAPTER 5. IN VITRO RESPONSES OF EQUINE COLONIC ARTERIAL AND VENOUS RINGS TO ADENOSINE TRIPHOSPHATE

5.1 Introduction

In horses, strangulating volvulus of the ascending colon is a disease characterized by colonic luminal obstruction and vascular occlusion, which results in colonic ischemia, mucosal necrosis and vascular thrombosis (1). In experimental models of complete arteriovenous occlusion of the ascending colon, colonic blood flow has been shown to remain significantly below baseline values for at least 4 hours after surgical correction (2). The disease is associated with a high mortality, which may be related to a sustained reduction of blood flow and hypoperfusion (due to increased vascular resistance) after surgical correction and continued ischemic injury. Endothelial damage in the colonic vasculature occurs subsequent to ischemia-reperfusion, and this damage can be exacerbated by endotoxin (3). Therefore, the sustained decrease in colonic blood flow may be associated with endothelial damage in the colonic circulation, leading to the release of vasoconstrictive agents and loss of endothelium-derived vasorelaxants, which subsequently may lead to vasoconstriction.

Extracellular purines have important and diverse effects on many biological processes, including regulation of vascular tone (4). Adenosine triphosphate (ATP) is principally an endothelium-dependent vasodilator that is rapidly metabolized and has a short duration of action (5). The vasodilatory effects of ATP are mediated primarily through activation of purinoreceptors located on both the endothelium and vascular smooth muscle (6). The purinergic 2Y (P2Y) receptors located on endothelial cells, when activated by ATP, couple to G proteins to activate phospholipase C leading to the formation of inositol-3-phosphate (IP_3) and mobilization of intracellular Ca^{2+} (4). Vasodilatation occurs by Ca^{2+}-dependent activation of endothelial nitric oxide synthase

(eNOS) with subsequent generation of nitric oxide (NO) and by generation of endothelial-derived hyperpolarizing factor (EDHF) (4). Generation of protein kinase C and subsequent phosphorylation of mitogen-activated protein kinase appears to be the pathway by which P2Y receptors on endothelial cells mediate prostacyclin synthesis and release to generate additional vascular relaxation (7,8). P2Y receptors are also present on vascular smooth muscle and mediate vasodilatation. The mechanism underlying smooth muscle cell relaxation is not known but may involve activation of K^+ channels (9). When ATP is degraded by ectonucleotidases into adenosine, an adenosine purinoreceptor (predominantly A_2) is activated, leading to vascular smooth muscle relaxation (10).

We have recently evaluated the local colonic and systemic hemodynamic alterations associated with intravenous infusion of a combination of ATP and magnesium chloride (ATP-$MgCl_2$) in clinically healthy, anesthetized, adult horses (11). Administration of ATP-$MgCl_2$ at an infusion rate of 0.3 mg of ATP/kg of body weight/min resulted in a significant decrease in colonic vascular resistance, principally via vasodilatation (11). These results suggest that ATP-$MgCl_2$ could have beneficial effects during low-flow conditions of the gastrointestinal tract by regulating vascular tone.

Since endothelial damage has been demonstrated to occur during colonic ischemia and reperfusion, the effects of ATP on regulation of vascular tone may be diminished or abolished owing to loss of endothelial-derived vasorelaxants, specifically NO. The purpose of this study was to evaluate the effects of ATP on vasomotor tone (specifically the vasodilatory response) of isolated equine colonic arterial and venous

rings in the presence and absence of endothelium and in the presence of a nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME). We hypothesized that ATP would cause vasodilatation in a concentration-dependent manner in both colonic arteries and veins. Secondly, the magnitude of the vasodilatory response in the arteries would be significantly attenuated with endothelial removal or the addition of L-NAME. Finally, we predicted that there would be no significant differences between veins with intact endothelium, denuded endothelium and those treated with L-NAME. Because veins have been shown to produce a weak response, compared with arteries, during endothelium-dependent relaxation (12) and the predominant site of action of nitric oxide is arterial resistance vessels (13), endothelium removal or blockade of nitric oxide in veins should minimally affects the vasodilatory response.

5.2 Materials and Methods

5.2.1 Horses - This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Segments of mesenteric vessels were collected from the left ventral colon of fourteen adult horses destined for euthanasia for reasons unrelated to cardiovascular or gastrointestinal tract diseases. Horses were deemed free of gastrointestinal and vascular disease and were euthanatized with an overdose of sodium pentobarbital[a] (100 mg/kg, IV). The vessels were collected and placed in chilled, oxygenated (95% O_2 and 5% CO_2) Tyrode's solution until used. The composition of Tyrode's solution is: 136.87 mM NaCl; 2.68 mM KCl; 11.90 mM $NaHCO_3$; 5.55 mM Dextrose; 1.81 mM $CaCl_2$; 1.07 mM $MgCl_2$; and 0.36 mM NaH_2PO_4.

5.2.2 Experimental design - The colonic artery and vein were cleansed of excess connective tissue and cut into 4-mm wide rings. Each ring was placed in an organ bath containing oxygenated Tyrode's solution at 37 °C. One side of the vessel ring was fixed to the floor of an organ bath and the other side attached to a force-displacement transducer[b] which was interfaced with a polygraph[c] (14,15). An initial tension of 2 g was applied to the rings, which were allowed to equilibrate for 45 minutes. Preliminary studies and previous reports from our laboratory have demonstrated that 2 g tension applied to equine colonic vessels resulted in optimum vessel responsiveness (14,16). The bath solutions were replaced at 15-minute intervals and tension readjusted to 2 g each time, except following the final wash.

5.2.2.1 Trial I - Arteries and veins (n=7 horses) with intact endothelium (endo +) and endothelium that was removed by gentle mechanical debridement (endo -) (16,17) were used. Sixteen tissue baths were used during this phase of the study. Two runs were performed for each horse in order to accommodate the 4 vessel type combinations (artery endo + and endo - and vein endo + and endo -). The vessel types were randomly assigned to each run for each horse. After equilibration, each ring was precontracted with a single dose of 10^{-7} M and 1.8×10^{-8} M endothelin-1 (ET-1)[d] for arteries and veins, respectively (Table 5.1). The EC_{50} (concentration required to produce 50% maximum contraction) values for colonic arteries and veins are 2.3×10^{-7} M and 6.7×10^{-8} M, respectively (18). The doses of ET-1 used in the present study were selected based on results of the study by Venugopal and colleagues (18) and from pilot studies in our laboratory. The selected doses produced a minimum of 500 mg of

Table 5.1 - Mean (±SEM) mg of stable contraction and mg of stable contraction/mg of vessel dry weight induced by endothelin-1(10^{-7} M for arteries and 1.8 x 10^{-8} M for veins) for colonic arterial (A) and venous (V) rings with (+) and without (-) intact endothelium (Trial I) or in the presence of 10^{-4} M N^{ω}-nitro-L-arginine methyl ester (LN) (Trial II). No statistical comparisons were performed on this data.

Vessel Type	Trial	mg Tension	mg Tension/mg Dry Weight
A+	I	1322±105.2	156.0±22.06
A-	I	2433±178.0	315.2±42.9
LNA+	II	1319±101.2	167.1±9.9
V+	I	1936±136.7	155.1±16.6
V-	I	1445±105.0	142.5±13.0
LNV+	II	2624±232.4	265.5±33.5

contraction, which was the minimum response that was stable, as well as relatively consistent responses in all vessel types utilized. Vessel rings that did not attain 500 mg of tension were rejected. Stock solutions of ET-1 were prepared in distilled water (10^{-2} M) and stored in 70-µl aliquots at -70 °C until used. On the day of the experiment, the ET-1 stock solution was thawed and appropriate dilutions were made with distilled water. When the contractile response reached a plateau, a non-cumulative concentration (10^{-8} to 10^{-3} M) response curve for ATP[e] was determined over 30 minutes for each vessel type. The ATP was prepared in distilled water immediately prior to performing the concentration-response curve. Additionally, 2 rings for each vessel type received only ET-1 to serve as a time controls.

5.2.2.2 Trial II - Arteries and veins (n=7 horses) with intact endothelium were obtained from a different group of horses than those used in Trial I. Eight tissue baths were used during this phase of the study. Similar to Trial I, 2 runs were performed on each vessel type (artery endo + and vein endo +), with the order randomized for each horse. The vessels were incubated with freshly prepared 10^{-4} M N^{ω}-nitro-L-arginine methyl ester (L-NAME)[f] for 30 minutes before determining the non-cumulative concentration-response relationship for each dose of ATP. The same concentrations of ATP were used as in Trial I. Similar to Trial I, the ATP and L-NAME were prepared fresh daily. For each vessel type, one ring received only ET-1 to serve as a time control. Additionally, one ring from each vessel type was treated with 10^{-4} M ATP in the absence of L-NAME to serve as a positive control in order to compare whether significant differences in relaxation response to 10^{-4} M ATP existed between vessels used in Trial I and II.

Separate arterial and venous rings from each horse were prepared (endo + and endo - for Trial I and endo + for Trial II) and placed in neutral-buffered formalin. The vessels rings were processed and stained with hematoxylin and eosin. Cross-sectional segments were examined histologically to evaluate the integrity, or lack thereof, of the endothelial and smooth muscle layers.

5.2.3 Data processing and statistical methods - The percentage relaxation for each vessel type was calculated based on the relaxation from the stable contraction induced by ET-1 to the resting tension, which was considered 100%. The percentage relaxation was calculated at 1, 3, 5, 7, 9, 11, 13 and 15 minutes after application of the ATP. Fifteen minutes was selected as our end-point because the contraction induced by ET-1 in the time-control vessels was stable during this time period, which indicates that any relaxation that occurred during that time was an effect of the ATP and not secondary to loss of the pre-contraction generated by ET-1. A curve was generated from these points and the area under the curve (AUC) was estimated using the trapezoid method (19). The AUC represents the integrated percentage relaxation over time and thus is represented by the units percentage relaxation time (% minutes). Only the area above the x-axis (relaxation) was included in the calculation.

The AUC was considered continuous. The AUC followed a normal distribution based on the Shapiro-Wilk statistic with failure to reject the null hypothesis of normality at $p \leq 0.05$.

The AUC was evaluated using a fixed effect linear model assuming a nested factorial design. To account for the fact that different vessel types were performed on different horses, the model was set up to include a fixed effect of trial. Trial I included

vessel types A+, A-, V+, and V-. Trial 2 included vessel types LNA+ and LNV+. Thus, vessel type was nested within trial with concentration (the repeated effect) factored over vessel type. The interaction term of vessel type and concentration was used as the error term to test for significant main effects. Where there were significant effects of concentration and of the interaction of vessel type and concentration at $p \leq 0.05$, selected ad hoc comparisons were made, using least square means. Comparisons were made within vessel types for all concentrations to the ET-1 control, maintaining an overall type I error of 0.05. Comparisons were made among vessel types at concentrations 10^{-3} M and 10^{-4} M, maintaining an overall type I error of 0.05. Thus, unless specified, p is $\leq$ 0.05 where a significant difference is noted.

To confirm that the horses of each trial behaved similarly, the AUC for 10^{-4} M ATP concentration for vessel types in Trial I were compared to 10^{-4} M ATP concentration (without L-NAME) in Trial 2 using the methodology described above. PROC UNIVARIATE, PROC MIXED and PROC GLM[8] were used for all analyses.

5.3 Results

Histologic evaluation of each representative vessel ring indicated the presence of intact endothelium and smooth muscle in endo + vessels and complete removal of endothelium without damage to the smooth muscle in endo - vessels. The data from Trial I and II studies demonstrated relaxation of colonic arteries and veins with and without intact endothelium and those pre-treated with L-NAME in response to ATP (Table 5.2; Figure 5.1 and 5.2). Concentrations of ATP from 10^{-6} to 10^{-3} M induced a concentration-dependent initial, rapid, and transient contraction followed by relaxation

Table 5.2 - Mean (±SEM) % maximum relaxation and % maximum attenuation for colonic arterial (A) and venous (V) rings with (+) and without (-) intact endothelium or in the presence of 10^{-4} M N^{ω}-nitro-L-arginine methyl ester (LN) in response to 10^{-4} M or 10^{-3} M ATP over a 15-minute time period. The % maximum attenuation is based on the relative change between (-) and (LN) vessels compared to the (+) vessels. A negative value indicates less relaxation and a + value indicates more relaxation. The time to attain maximum relaxation may vary among and between vessel types. No statistical comparisons were performed on this data.

	10^{-4} M ATP		10^{-3} M ATP	
Vessel Type	**% Maximum Relaxation**	**% Maximum Attenuation**	**% Maximum Relaxation**	**% Maximum Attenuation**
A+	36.71±4.39		56.90±5.94	
A-	24.42±9.16	-30.06±22.19	33.99±12.52	-45.17±18.45
LNA+	36.98±9.29	-1.425±18.54	75.09±14.76	33.80±25.62
V+	56.94±9.47		59.46±3.55	
V-	36.65±5.98	–29.16±12.58	41.28±4.15	-30.31±6.47
LNV+	34.30±7.60	-29.52±17.98	62.47±5.85	6.35±10.72

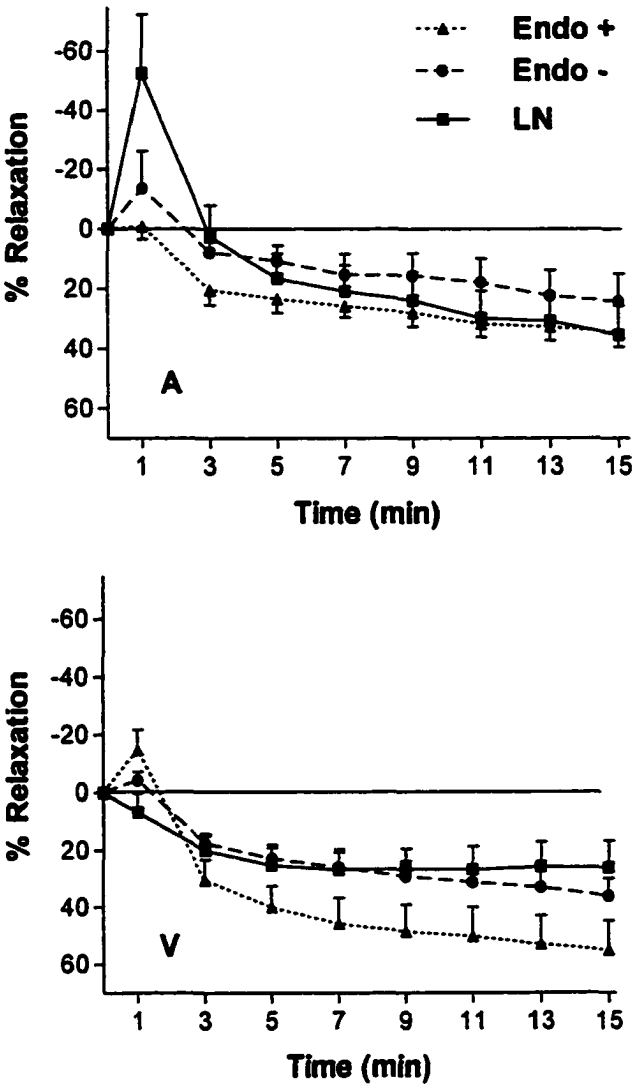

Figure 5.1 - Time-response curve for mean (±SEM) percent relaxation of equine colonic arterial (A) and venous (V) rings to 10^{-4} M ATP that were precontracted with endothelin-1. Because percentage relaxation was not used in the study to identify significant differences within and between vessel type/treatment, no statistical comparisons were made.

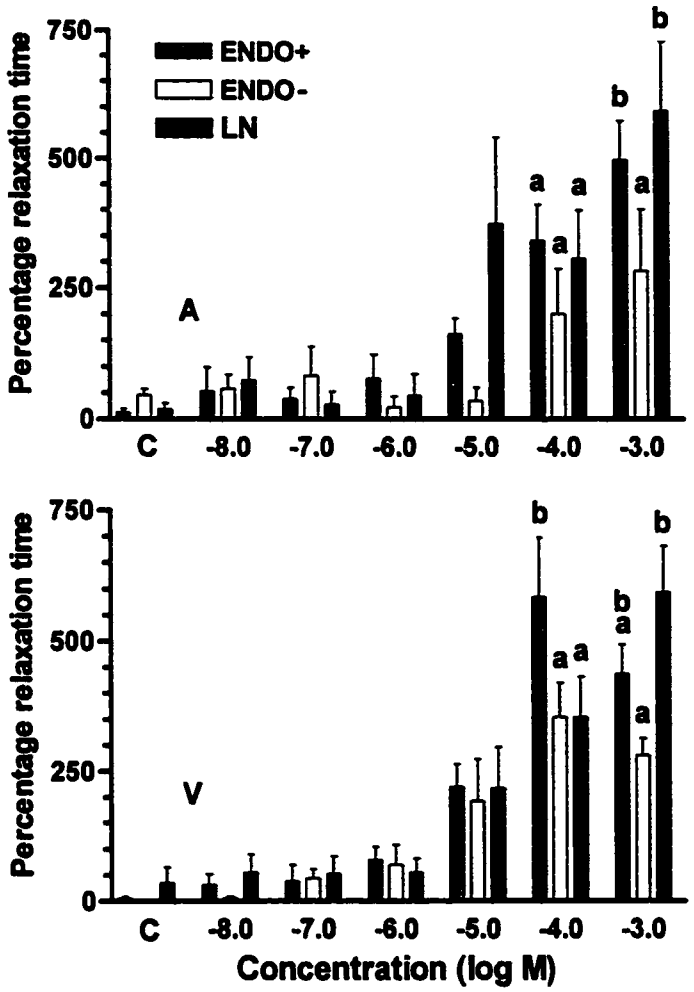

Figure 5.2 - Mean (±SEM) percent relaxation time (area under the curve) of equine colonic arterial (A) and venous (V) rings after 15 minutes of exposure to ATP (10^{-8} to 10^{-3} M) following precontraction with endothelin-1. Endo + = intact endothelium; Endo - = endothelium removal; LN = endo + vessels incubated with 10^{-4} M N^{ω}-nitro-L-arginine methyl ester (L-NAME); C = time control (ET-1 only). Within group comparisons were made at 10^{-4} M and 10^{-3} M ATP. Different letters (a,b) indicate significant differences ($p \leq 0.05$) between vessel types within that concentration of ATP.

in all vessel types. There was a significant effect of ATP concentration, vessel type and their interaction on the percentage relaxation time (Figure 5.2). There were no significant differences between the time control vessel rings. There was no significant difference in the time control vessel rings (ET-1 only) during the 15-minute evaluation period.

5.3.1 Trial I - At 15 minutes, ATP concentrations of 10^{-4} M and 10^{-3} M caused significant relaxation, for endo + arteries and veins and endo - veins. Significant relaxation for endo - arteries was only observed at 10^{-3} M ATP. Endo + and endo - vessels at 10^{-3} M and 10^{-4} M ATP revealed significant differences in relaxation from the stable contraction induced by ET-1 in arteries and veins (Figure 5.2). When the relaxation response between arteries and veins was compared, there were no significant differences in relaxation between endo + arteries and veins at 10^{-3} M ATP and endo - arteries and veins at 10^{-3} M and 10^{-4} M ATP. However, endo + arteries relaxed significantly less than endo + veins at 10^{-4} M ATP.

5.3.2 Trial II - There was no significant difference in relaxation response between the vessels treated only with 10^{-4} M ATP from Trial I and Trial II. At 15 minutes, significant differences in relaxation from the stable contraction induced by ET-1 was observed for L-NAME-treated arteries and veins at 10^{-4} M and 10^{-3} M ATP. There were significant differences in percentage relaxation time between endo - arteries and veins (Trial I) and their L-NAME-treated arteries and veins (Trial II) at 10^{-3} M ATP and endo + veins (Trial I) and their L-NAME-treated counterpart (Trial II) at 10^{-4} M ATP (Figure 5.2). There were no significant differences in relaxation response between L-NAME-treated arteries and veins at 10^{-3} or 10^{-4} M ATP.

5.4 Discussion

The results of this study yielded several important findings. First, ATP can overcome ET-1-induced contraction. Second, the relaxation response of equine colonic arterial and venous rings to ATP is dose-dependent. Third, removal of endothelium attenuates but does not eliminate the relaxation response to ATP in both colonic arteries and veins. Fourth, the contribution of NO to the relaxation response is minimal and appears to be more appreciable in veins. Finally, the vascular response to ATP is biphasic at high concentrations (initial transient contraction followed by slow relaxation).

In contrast to numerous studies that have demonstrated that the mechanism of ATP-induced vasodilatation is principally mediated by NO, the results of the present study suggest that NO does not appreciably contribute to the endothelium-dependent component of the relaxation response to ATP in normal equine colonic vessels. A study by Simonsen and colleagues evaluating the effect of ATP on vasomotor tone of lamb isolated coronary small arteries demonstrated that mechanical removal of the endothelium, but not inhibition of NOS, partially reduced the relaxations elicited by exogenously added ATP (20). The results indicated that ATP relaxed lamb coronary small arteries through receptors located on both smooth muscle and endothelial cells. Furthermore, the results exclude the possibility that NO is the mediator of the endothelial component of the relaxations to ATP in that particular vessel preparation (20). The results of the present study correlate with results from the study by Simonsen and colleagues and our in vivo study (11), which failed to identify a significant increase

in colonic arterial and venous plasma NO concentrations in horses administered ATP-$MgCl_2$, despite the development of profound colonic vasodilatation.

Several possible explanations for the observations noted in the present study and the in vivo colon study (11) exist. Although not evaluated, prostacyclin may play an important role in endothelium-dependent ATP relaxation in equine colonic arterial and venous rings. Further studies using indomethacin to block prostacyclin synthesis would be necessary to elucidate the role of prostacyclin in ATP-induced vasodilatation. Additionally, activation of P2Y receptors with subsequent IP_3 formation and Ca^{2+} mobilization can lead to the synthesis of endothelium-derived hyperpolarizing factor, which may also contribute to the vascular relaxation response (4).

Another possible explanation for the apparent lack of involvement of NO in the vasodilatory response to ATP would be that we did not completely inhibit NOS. The dose of L-NAME used in this study (10^{-4} M) is comparable to other studies that have evaluated the role of NO in the vascular response in horses (15,17,21). Concentrations of L-NAME ranging from 10^{-6} M (21) to 10^{-3} M (17) have been used and shown to be effective in blocking NO production. In our initial pilot studies, we compared colonic vascular responsiveness to acetylcholine in the presence of 10^{-5} M and 10^{-4} M L-NAME. Our results indicated that 10^{-4} M yielded better blockade than 10^{-5} M (unpublished data). Furthermore, results from an in vivo L-NAME pharmacokinetic study in horses demonstrated that systemic and pulmonary hypertension rapidly develops following an IV bolus injection of L-NAME (40 mg/kg) (personal communication). Therefore, incomplete blockade of NOS is unlikely to explain the results obtained in this study.

One observation that was not expected was the blockade of the endothelium-dependent component of the relaxation response of colonic veins to 10^{-4} M ATP when incubated with 10^{-4} M L-NAME. The same blockade was not observed with 10^{-3} M ATP or in the artery preparations. This finding is in contrast to studies that have demonstrated that the predominant site of action of nitric oxide is arterial resistance vessels (13). One reason to explain these findings would be that at physiologic concentrations of ATP (10^{-4} M), endothelium-dependent relaxation in colonic veins is NO-mediated. Although speculative, when superphysiologic concentrations of ATP (10^{-3} M) were used, other endothelium-dependent vasodilators (prostacyclin and EDHF) may become more important. The reason why L-NAME blocked the endothelium-dependent component of the relaxation response to 10^{-4} M ATP in colonic veins but not arteries in the present study is not known and warrants further investigation.

Endothelin-1 was selected as our precontractile agent for several reasons. First, since ATP historically is a slowly relaxing agent, we wanted a precontractile agent that would sustain the contraction for a minimum of 15 minutes in order to more fully evaluate the effects of ATP on in vitro vasomotor tone. Secondly, since ET-1 is the most potent vasoconstricting agent presently identified and it has been implicated as a cause of decreased blood flow that commonly occurs during ischemia (22), we wanted to determine whether ATP could overcome ET-1-induced vasoconstriction. Previous studies in our laboratory demonstrated that a consistent, prolonged contraction could be achieved in colonic arteries and veins with ET-1 (18) and our pilot studies showed that ATP could overcome this ET-1-induced vasoconstriction (unpublished data).

In order to evaluate relaxation responses in vascular ring preparations, the rings must first be contracted to establish tension so a relaxation response can be detected. One limitation of in vitro relaxation studies is the inability to control the amount of stable contraction induced by a vasoconstricting agent or electrical field stimulation. The magnitude of the response to a vasodilating agent may vary depending upon the amount of initial tension generated. The effect of various tensions on the relaxation response to ATP was not evaluated in the present study. Therefore, interpretation of results for the different vessel types used in this study may vary if the relaxation response is significantly influenced by the initial tension generated.

In this study, we used an unstable form of ATP rather than a stable analog. The ATP used in the present study was the same ATP that we have been using in our in vivo studies (ATP combined with magnesium chloride) (11,23). Since significant vasodilatation occurs in vivo with IV administration, the mechanism of the vasodilatory response was important to determine. Particularly, determining whether the form of ATP that would be used in clinical situations could cause vasodilatation in the absence of endothelium, as could occur during colonic ischemia, was vital to future studies evaluating the efficacy of ATP during colonic ischemia.

Unlike our in vivo studies where we used ATP-$MgCl_2$ (11,23), we only evaluated the in vitro effects of ATP in the present study. Magnesium has been reported to be a potent vasodilating agent and has been demonstrated to potentiate vasodilatation when combined with ATP in vivo (24). However, we do not believe that magnesium was a major contributor to the vasodilatation response observed in the in vivo studies. In an in vitro pilot study, we documented that addition of $MgCl_2$ (10^{-12} to

10^{-4} M) to ATP (equimolar concentrations) did not enhance the relaxation response of equine colonic arterial and venous rings (with intact and denuded endothelium) precontracted with endothelin-1, compared with ATP alone (unpublished data). Additionally, $MgCl_2$ alone did not cause appreciable relaxation of colonic vascular rings (with intact and denuded endothelium) precontracted with endothelin-1 (unpublished data).

Because the ATP used in the present study is subjected to degradation by tissue ectonucleotidases to adenosine, it was not possible to determine whether ATP or adenosine mediated the smooth muscle component of the vascular relaxation response. As previously mentioned, both P2Y receptors, which respond to ATP, and A_2 receptors, which respond to adenosine, have been identified on vascular smooth muscle (4). Additional studies using specific adenosine blockers would need to be performed in order to determine what percentage of the smooth muscle relaxation response was attributable to ATP and what percentage was attributable to adenosine.

Numerous studies have documented that high concentrations of ATP cause a transient vascular smooth muscle contraction followed by a slow, sustained relaxation. Similar results were observed in this study. The mechanism of ATP-induced contraction is via activation of P2X receptors located on smooth muscle cells (25,26). Ligand binding to the P2X receptor results in the rapid, non-selective passage of cations (Na^+, K^+, Ca^{2+}) across the cell membrane resulting in an increase in intracellular Ca^{2+}, membrane depolarization, and smooth muscle relaxation (25,26). Whether the same mechanism for ATP-induced vasoconstriction occurs in the horse is not known. The concentration-dependent initial, transient contraction induced by higher concentrations

of ATP combined with the lack of specific antagonists to block this response, complicates interpretation of the relaxation effects of ATP.

The vascular rings used in the present study were collected from normal horses. Mechanically removing the endothelium attempts to mimic the effect of diseases of the colon that lead to endothelial dysfunction. However, disease mechanisms not tested in this study could alter the vascular response to ATP, both in vivo and in vitro. Additionally, the responses and mechanisms induced by ATP might be different along the colonic vascular bed. The segments used in this study might not be predictive of responses of the microvasculature, which are important to the various components of the intestinal wall.

In the present study, exogenous ATP applied to both equine colonic arterial and venous rings precontracted with ET-1 in vitro causes a biphasic response characterized by initial, transient contraction followed by slow, substantial and sustained relaxation. The relaxation response is attenuated by endothelium removal but it is not inhibited by blocking NO production, suggesting a relaxation mechanism other than NO. Further studies evaluating the role of prostacyclin and adenosine in the relaxation response need to be performed to further define the mechanism of action of ATP-induced vasodilatation. Since vasodilatation does occur in the absence of endothelium, further studies evaluating the efficacy of exogenously-administered ATP (in the form of ATP-$MgCl_2$) to modulate vasomotor tone during colonic ischemia warrants investigation.

5.5 Personal Communication

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5.6 Product Information

[a]Beuthanasia-D Special, Schering-Plough Animal Health Corp., Union, NJ.

[b]Model FT03 force-displacement transducer, Grass Medical Instruments, Quincy, Mass.

[c]Model 7D polygraph, Grass Medical Instruments, Quincy, Mass.

[d]Endothelin-1 E-7764, RBI/Sigma, Natick, Mass.

[e]Adenosine 5'-triphosphate disodium salt A3377, Sigma-Aldrich Inc, St. Louis, Mo.

[f]N^{ω}-nitro-L-arginine methyl ester N5751, Sigma-Aldrich Inc, St. Louis, Mo.

[g]SAS v 8.0, SAS Institute, Cary, NC.

5.7 References

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CHAPTER 6. QUANTITATION OF ADENINE NUCLEOTIDES IN EQUINE COLONIC MUCOSAL TISSUE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

6.1 Introduction

Liquid chromatography (LC) refers to any chromatographic procedure in which the moving phase is a liquid (1). Since LC is not limited by sample volatility or thermal stability, it is ideally suited for the separation of macromolecules and ionic species of biomedical interest, labile natural products, and a wide variety of other high-molecular-weight and/or less stable compounds. Chromatographic separation is the result of specific interactions between sample molecules and the stationary and moving phases. These interactions enhance the ability to control and improve separation. Numerous column packings (stationary phases), solutions (moving phases), and detectors are available in LC, which allow a wider variation of selective interactions and greater possibilities for separation. Also, separated fractions can be easily collected. Recovery is quantitative and separated sample components are readily isolated for identification by supplementary techniques or other use. High performance liquid chromatography (HPLC) refers to the use of high-pressure pumps to establish a controlled, rapid solvent flow which results in more reproducible operation and, therefore, greater accuracy and precision (1).

Gastrointestinal mucosal cells are highly metabolically active and, therefore, require large quantities of adenosine triphosphate (ATP) for maintenance of normal cellular function (2). Because of this, the mucosal layer is principally affected during ischemia (2). One proposed mechanism for the increase in cellular permeability observed during ischemia is decreased endogenous production of ATP. In an experimental model of ascending colon volvulus in ponies, mucosal ATP content diminished 92% during ischemia and recovered to only 44% of control values upon

reperfusion (3). In cultured renal and intestinal epithelial cell lines, decreasing cellular ATP content by inducing chemical hypoxia results in rapid opening of tight junctions, which increases paracellular permeability (4,5). Therefore, mucosal ATP depletion, which occurs during gastrointestinal ischemia, may cause rapid opening of tight junctions leading to enhanced absorption of bacteria and endotoxin into the splanchnic circulation. These findings suggest that increased metabolic support to ischemically injured colonic epithelium may be important in decreasing morbidity and improving survival (3). Quantitation of adenine nucleotide content in equine colonic mucosal tissue, using HPLC analysis, will provide information regarding the relationship between ATP content, and its catabolites adenosine diphosphate (ADP) and adenosine monophosphate (AMP), and alterations in epithelial cell permeability that occur during ischemia. Furthermore, potentially increasing adenine nucleotide content in mucosal tissue using pharmacologic agents (ie. ATP-$MgCl_2$) to enhance mucosal cell recovery can be determined using this technique.

Tissue levels of ATP, ADP, and AMP have been separated by HPLC in numerous species and tissues (6). However, separation and quantitation of adenine nucleotides in equine colonic mucosal tissue has not been reported. The purposes of this study were to validate an established method for adenine nucleotide separation (6) in equine colonic mucosal tissue, to determine the inherent variability in the tissue and extraction method, and to determine the stability of ATP, ADP and AMP in the tissue across time.

6.2 Materials and Methods

6.2.1 Animals - The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Equine colonic mucosal tissue (20 mm x 300 mm strip) was obtained from one horse euthanatized (sodium pentobarbital,100 mg/kg, IV) for reasons unrelated to gastrointestinal tract disease. The tissue was harvested from the pelvic flexure region of the ascending colon immediately after euthanasia and placed in oxygenated Ringer's solution and transported to the laboratory. Composition of the Ringer's solution (mM) included: 114 NaCl, 5 KCl, 1.25 $CaCl_2$, 1.1 $MgCl_2$, 25 $NaHCO_3$, 0.3 NaH_2PO_4 and 1.65 Na_2HPO_4. The mucosa was separated from the submucosa in oxygenated Ringer's solution and divided into multiple parents sections. The sections were placed in cryotubes, immediately submersed in liquid nitrogen until frozen and then stored at -70 °C overnight.

6.2.2 Adenine nucleotide quantitation - Twenty four hours after collection, the frozen samples were lyophilized[a] (freeze-dried) and stored in a desiccator at -70 C until analyzed. The extraction method used in this study was adapted from a technique described by Wynants and colleague (6). On the day of analysis, frozen tissue samples were weighed (7-13 mg dry weight) and placed in individual 10-ml conical tubes on ice. Iced cold perchloric acid (1 ml of 0.6 N $HCLO_4$ /1.5 mg of tissue dry weight) was added to each sample, and the samples were homogenized, using a tissumizer[b]. The samples were centrifuged at 2,500 rpm (1,500 x g) at -10 C for 12 minutes. The tissue extracts (supernatant) were removed and 1,200 µL was placed in individual 5-ml conical tubes on ice. The samples were neutralized with 800 µL of iced cold potassium bicarbonate (1.0 M) and then centrifuged at 2,000 rpm (1,000 x g) at -10 C for 15 minutes.

Neutralized samples were filtered through a nylon filter[c] (0.45 *um*), and the ATP, ADP and AMP were separated by HPLC, using an Alltech Adsorbosphere C18 5 *um* column[d]. The column was eluted by gradient, using 0.15M ammonium phosphate and methanol/acetonitrile (50:50), at a gradient of 100% to 93% of ammonium phosphate over 10 minutes. Detection was performed by a diode-array detector[e] set at a peak wavelength of 260 nm. The run time for each sample was 10 minutes with a 5 minute post-run period. The peaks were quantified by area under the curve and compared with peak areas of known standards of ATP, ADP and AMP. The limit of quantitation on the column was 0.05 μg/ml.

6.2.3 Instrument Variability (Phase I) - One tissue sample from one parent segment was processed for adenine nucleotide quantitation, using the above described technique. The single sample was loaded into the instrument and quantified 18 consecutive times to determine the inherent variability of the instrument.

6.2.4 Sample/Method Variability (Phase II) - Six individual small samples were obtained from one parent segment and processed individually for adenine nucleotide quantitation, using the above described technique. The samples were analyzed in duplicate to determine the variation inherent in the sample preparation and extraction method.

6.2.5 Tissue Storage Stability (Phase III) - Multiple parent segment of tissue were crushed, using a chilled mortar and pestle, and combined to improve the homogeneity of the individual samples. Three samples of ground tissue were analyzed in duplicate for adenine nucleotide content, using the above described technique. The analyses were performed at time 0 (2 days after collection; 1 day after lyophilization)

and again at time 7, 14, 21, 32, 35, 46, and 54 (days after the initial adenine nucleotide quantitation) to determine stability of adenine nucleotides in lyophilized tissue across time.

6.2.6 Statistical Analyses - The variability inherent to the HPLC analysis of ATP, ADP and AMP on a single sample run 18 times was summarized as the coefficient of variation (CV). The effect of sample and analysis run on the measurement of ATP, ADP and AMP was analyzed using a fixed effect, two factorial general linear model. Additionally, the inter-run coefficient of agreement was calculated. The effect of time on ATP, ADP and AMP stability was analyzed, using a fixed effect, general linear model accounting for the repeated measurements across time. Initial analysis accounted for the effect of time, tissue and run. For ATP and ADP, there was no effect of run and the data for each run was combined and re-analyzed with each run considered a replicate. Comparisons were made to baseline (time 0) using Dunnett's test for multiple comparison maintaining an experiment-wise type I error of 0.05. For AMP, comparisons were made to time 7 since there were only two measurements at time 0. For all analyses, a $p \leq 0.05$ was considered significant. A statistical software package[f] was used for the analyses.

6.3 Results

The CV for the instrument was 5.89% for ATP, 7.68% for ADP and 9.73% for AMP. For sample/method variability, there was a significant effect of tissue sample ($p=0.001$) for ATP, ADP and AMP and a significant effect of run ($p=0.034$) for AMP. The inter-run coefficient of agreement (Pearson's ρ) was 0.99 for ATP and ADP and 0.98 for AMP. For the tissue storage stability, there was no significant effect of tissue

or time for ATP and ADP. However, there was a significant effect of time ($p=0.0135$), but not tissue, for AMP (Fig. 6.1).

6.4 Discussion

The CV for the instrument used in this study was less than 10% for all nucleotides measured. The greatest variation during Phase I occurred in AMP quantitation and this was associated with diminished resolution of the AMP peak. Resolution relates to the relative separation of molecules and can affect the accuracy of band area measurement (1). Minor differences in band area measurement could account for the higher CV for AMP observed in this study. Another factor that can affect CV is the stability of the extracted nucleotides. The total run time for the 18 samples was 285 minutes. Based on pilot work performed during the validation procedure, no appreciable degradation of ATP, ADP and AMP was observed over a 13-hour run time. However, in this study we were unable to determine what portion of the CV could be attributed to the instrument and what portion, if any, could be attributed to nucleotide instability .

The inconsistency in adenine nucleotide content in mucosal samples during Phase II of this study was related to the sampling method rather than the extraction method. Small samples were broken off from the parent sample after lyophilization and then analyzed. Differences in cell type and cell content may exist across the parent sample, which could account for the variation observed in this study. For Phase III the parent segments were ground in a chilled mortar prior to analyses to improve homogeneity. Results of statistical analyses of Phase III data revealed that no tissue/method effect was present, indicating that we were able to eliminate variability.

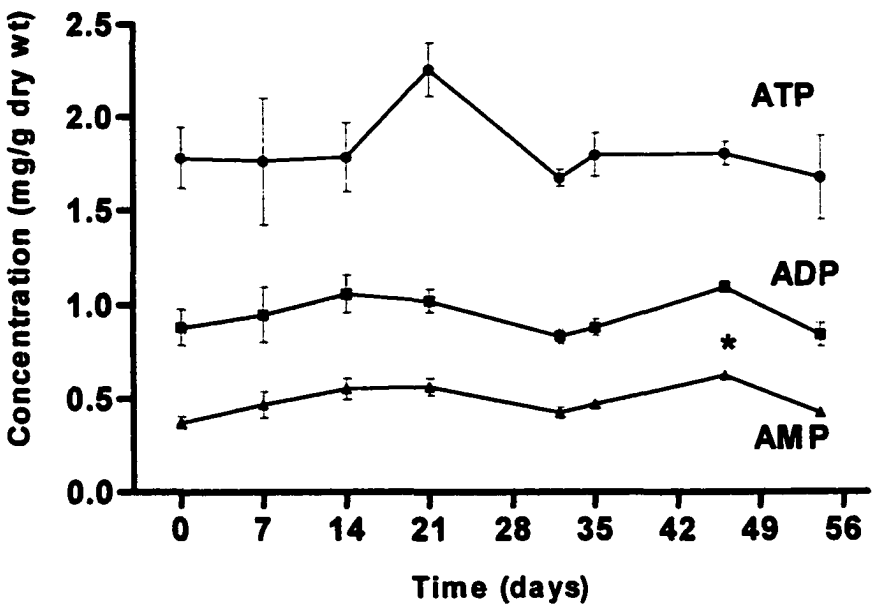

Figure 6.1 - Mean (± SEM) adenine nucleotide content in equine colonic mucosal tissue across time. * Significant difference ($p \leq 0.05$) from day 7.

Based on these results, we believe the variability in our data was a result of tissue variability and not extraction method variability. Currently, the authors recommend that each frozen sample be ground prior to lyophilization. Another option to minimize the variability in the data, although less favorable, would be to increase the number of replicates analyzed. The results of this study indicate that a minimum of 100 replicates would be required.

Increases in retention time (RT) for AMP occurred during the analyses. Reasons for generalized increases in RT include: low mobile-phase flow rate; low column temperature; improper gradient setting; and increased column activity resulting in the system not being equilibrated (1). However, AMP was the only nucleotide affected in this study. During the analysis, material from the extracted samples accumulates at the head of the column. As the number of samples analyzed increases, the quantity of accumulated material subsequently increases. Binding of AMP to the material may delay its release from the column since the solubility of the AMP alone is not the same as the solubility of bound AMP. This could result in an increase in RT. In this study, the majority of RT problems were observed toward the middle to end of a run, which would allow sufficient time for material to accumulate on the column and bind AMP. In an attempt to decrease the amount of material at the head of the column, a pre-filter and guard cartridge were utilized in this study. The authors recommend that the maximum number of samples analyzed in one run be limited to 12 in order to maintain good resolution of the adenine nucleotide peaks.

The resolution of the chromatogram was excellent for the ATP and ADP, however, diminished resolution of the AMP peak was observed across time. As stated

previously, binding of AMP to material on the head of the column can affect resolution quality. Another factor that can contribute to poor resolution is the age of the column. In this study, the column used contained silica. The silica bonds can break down across time and form free silica hydroxyl groups. These groups can interact with the molecule of interest resulting in chromatographic peaks that demonstrate poor resolution. Finally, the mobile phase used in this study contained extremely high concentrations of salts which accumulate on the column and can affect resolution. The authors recommend that the column be washed thoroughly with water (forward and reverse wash) between runs to prevent accumulation of salts which can lead to damage to the column and instrument.

In conclusion, quantitation of adenine nucleotide content in equine colonic mucosal tissue, using HPLC, provided reproducible results when the tissue was ground into a homogeneous sample. The stability of the adenine nucleotides measured in this study following lyophilization within 24 hours of sample collection and stored in a desiccator at -70 °C was greater than 30 days. The extraction method was simple and required minimal equipment to accomplish (refrigerated centrifuge and a tissumizer). The RT for the 3 nucleotides analyzed in this study was under 10 minutes, which allows analysis of multiple samples in one run.

6.5 Product Information

[a]FreeZone 4.5 liter freeze dry system, Model 77510, Labconco Corporation, Kansas City, MO

[b]Ultra-turrax T25, Tekmar Corporation, Cincinnati, OH

[c]Nalgene 4-mm syringe filters #176-0045, Nalge Company, Rochester, NY

[d]Absorbosphere C18 5U column, Direct-connect prefilter kit #28689, All-guard cartridge system #96041, Alltech Associates, Deerfield, IL

[e]HP 1090 liquid chromatograph, Agilent Technologies, Wilmington, DE

[f]PROC MEANS, PROC GLM, PROC REG, SAS version 6.12, SAS Institute, Cary, NC

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CHAPTER 7. IN VITRO DEPLETION OF ADENINE NUCLEOTIDES IN EQUINE COLONIC MUCOSAL TISSUE AFTER EXPOSURE TO AN ELECTRON TRANSPORT INHIBITOR

7.1 Introduction

Because gastrointestinal mucosal cells are highly active metabolicallyand require large quantities of ATP for maintenance of normal cellular function, the mucosal layer is principally affected during ischemia (1). Increased cellular permeability, which occurs during ischemia and hypoxia, leads to translocation of bacteria and endotoxin from the gastrointestinal tract lumen into the splanchnic and systemic circulation. One mechanism that may be involved in the increase in gastrointestinal mucosal permeability during ischemia is decreased endogenous synthesis of ATP.

The epithelial intercellular permeability barrier is maintained largely by the tight junction (TJ) (2). Tight junctions are continuous, circumferential, intercellular contacts between epithelial cells, which are responsible for generating and maintaining a passive permeability barrier that regulates paracellular solute flow (3). In vitro studies have demonstrated that a correlation exists between cellular ATP content and TJ integrity (4). When cells in culture are experimentally depleted of ATP, disruption of signaling processes occurs resulting in paracellular barrier dysfunction (4). During ischemia and subsequent reoxygenation, several lesions occur in epithelial cells including mispolarization of some membrane proteins, perturbation of the actin cytoskeleton and disruption of the permeability barrier (5,6). These lesions can be reproduced in cell culture models for hypoxia-reoxygenation injury, using agents that deplete cellular ATP (4).

Several reversible in vitro models which produce chemical hypoxia or oxygen deprivation have been developed to deplete cellular ATP content. Chemical hypoxia can be induced with antimycin A (AA, oxidative inhibitor) (4), sodium cyanide (7), or a

combination of AA plus 2-deoxy-glucose (glycolytic inhibitor) (2). Oxygen deprivation can be induced by replacing oxygen with nitrogen gas (8). One of the most widely utilized methods to produce chemical hypoxia with subsequent ATP depletion is AA. Antimycin A interferes with electron flow from cytochrome b_H in cytochrome reductase, thereby inhibiting the electron transport chain and ATP synthesis (9).

Ascending colon volvulus is a common cause of gastrointestinal tract ischemia in horses (10). In an experimental model of ascending colon volvulus in ponies, mucosal ATP content was found to diminish 92% after ischemia and recovered to only 44% of control values upon reperfusion (11). The depletion of mucosal ATP content may lead to rapid opening of TJs and increased passage of bacteria and endotoxin through the paracellular pathway and into the splanchnic circulation. Early reestablishment of cellular ATP content following an intestinal ischemic event may help minimize mucosal barrier disruption with subsequent passage of toxic mediators into the splanchnic circulation, thereby decreasing morbidity and mortality.

Based on this information, we hypothesized that AA would deplete equine colonic mucosal adenine nucleotide content and upon its removal, adenine nucleotides would be repleted. The purposes of this study were to evaluate the effect of AA on equine colonic mucosal adenine nucleotide content, and secondly, to determine the stability of adenine nucleotides in an in vitro system. The development of an in vitro reversible model of ATP depletion for equine colonic mucosal tissue will enable investigators to characterize the role of ATP in maintenance of cellular integrity. Additionally, methods to modulate TJ integrity during adenine nucleotide depletion can be evaluated.

7.2 Materials and Methods

7.2.1 Animals - The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Six horses euthanatized for reasons unrelated to gastrointestinal tract disease were used. Immediately following euthanasia (pentobarbital sodium[a],100 mg/kg, IV), a full-thickness tissue segment was collected from the pelvic flexure region of the left ventral colon. The tissue was rinsed of intestinal contents, using 0.9% NaCl, and transported to the laboratory in preoxygenated (95% O_2-5% CO_2), chilled Ringer's solution. Composition of the Ringer's solution (mM) was: 114 NaCl, 5 KCl, 1.25 $CaCl_2$, 1.1 $MgCl_2$, 25 $NaHCO_3$, 0.3 NaH_2PO_4 and 1.65 Na_2HPO_4. The tissue was pinned on a rubber surface, with mucosal side up, and submerged in oxygenated (95% O_2-5% CO_2) Ringer's solution at room temperature (20-22 °C). Mucosal segments (6 mm x 6 mm) were harvested and placed in organ baths. The segments were bathed in oxygenated (95% O_2-5% CO_2) Ringer's solution at a volume of 10 ml and temperature of 37 °C.

7.2.2 Experimental Design - Four different organ bath conditions were used (Table 7.1). All tissues were allowed an equilibration period of 15 minutes in the organ bath prior to collection of the baseline (t= 0) sample. The remainder of the samples were collected at 60, 120, 180 and 240 minutes after the baseline samples were obtained. Baths 1 through 4 contained Ringer's solution with 10 mM glucose[b] (SCRS). Baths 5 through 8 contained Ringer's solution with no glucose added (SFRS). Baths 9-12 contained Ringer's solution with no glucose added. After obtaining the baseline sample, antimycin A[c] (50 μM) was added to the bath solution (AA/D). Baths 13

Table 7.1 - Organ bath/tissue design. Segments (6 mm x 6 mm) of equine colonic mucosal tissue were placed in individual organ baths and bathed in oxygenated (95% O_2-5% CO_2) Ringer's solutions with the following treatments added at time = 0 minutes: SCRS - substrate-containing (10 mM glucose) Ringer's solution; SFRS - substrate-free Ringer's solution; AA/D - 50 μM antimycin A (AA) in SFRS; AA/R - AA removed and replaced with SCRS only; Frozen - tissue removed from organ bath and snap frozen in liquid nitrogen; NT - no tissue in organ bath.

Organ Bath	0 min	60 min	120 min	180 min	240 min
1	SCRS	Frozen	NT	NT	NT
2	SCRS	SCRS	Frozen	NT	NT
3	SCRS	SCRS	SCRS	Frozen	NT
4	SCRS	SCRS	SCRS	SCRS	Frozen
5	SFRS	Frozen	NT	NT	NT
6	SFRS	SFRS	Frozen	NT	NT
7	SFRS	SFRS	SFRS	Frozen	NT
8	SFRS	SFRS	SFRS	SFRS	Frozen
9	AA/D	Frozen	NT	NT	NT
10	AA/D	AA/D	Frozen	NT	NT
11	AA/D	AA/D	AA/D	Frozen	NT
12	AA/D	AA/D	AA/D	AA/D	Frozen
13	AA/D	AA/R	Frozen	NT	NT
14	AA/D	AA/R	AA/R	Frozen	NT
15	AA/D	AA/R	AA/R	AA/R	Frozen

through 15 contained Ringer's solution with no glucose initially (SFRS). After collection of the baseline sample, 50 μM AA was added (AA/D). After the 60-minute sample collection, the Ringer's/AA solution was removed and replaced with Ringer's solution and 10 mM glucose (AA/R).

7.2.3 Adenine nucleotide quantitation - At the appropriate time periods, tissues were removed from the baths, snap frozen in liquid nitrogen and stored in cryotubes at -70 C until lyophilized. Within 3 days of collection, the samples were lyophilized[d] and stored in a dessicator at -70 °C prior to high performance liquid chromatography (HPLC) analysis, which was performed within 10 days of sample collection. The method for adenine nucleotide quantitation in equine colonic mucosal tissue using HPLC has been described (12). Briefly, frozen lyophilized tissues were weighed (7-13 mg) and placed in individual conical tubes on ice. Ice-cold perchloric acid (1 ml of 0.6 N $HCLO_4$/1.5 mg of tissue dry weight) was added to each sample, and the sample tissues were homogenized, using a tissumizer[e]. Subsequently, the samples were centrifuged (1,500 x g) at -10 °C for 12 minutes, the supernatant removed, and 1,200 μL of the supernatant placed in individual conical tubes on ice. After neutralizing the samples with 800 μL of ice-cold potassium bicarbonate (1.0 M), they were centrifuged (1,000 x g) at -10 °C for 15 minutes. Neutralized samples were filtered (0.45 μm nylon filter[f]) and the ATP, ADP and AMP were separated by HPLC, using an Alltech Absorbosphere C18 5μm column[g]. The column was eluted by gradient, using 0.15M ammonium phosphate and methanol/acetonitrile (50:50), at a gradient of 100% to 93% of ammonium phosphate over 10 minutes. Detection was performed by a diode-array detector[h] set at a peak wavelength of 260 nm. The peaks were quantified by area

under the curve and compared with peak areas of known standards of ATP, ADP and AMP.

7.2.4 Statistical Analyses - The study was considered a completely randomized block design, blocked by tissue, with replication of each Treatment (n=2) within each block. All continuous data were evaluated for normality, using the Shapiro-Wilk statistic and considered to follow a normal distribution with failure to reject the null hypothesis of normality at $p \leq 0.05$. Non-normal data were transformed. The data were summarized and graphed as mean ± SEM.

The data were evaluated using a mixed effect, general linear model accounting for the random variance of Horse and repeated measurements on each horse. A two-sided hypothesis with $\alpha = 0.05$ was used to determine significance of the fixed effects. Where there were significant fixed effects, including interactions, selected adjusted least squares means comparisons maintaining an experiment-wise error of $\alpha = 0.05$ were made within treatments to describe the changes over time. Thus, where a difference was noted, the p-value was ≤ 0.05. Treatments that behaved differently over time were implied to be different. SAS version 8.0[i] was used for the analyses.

7.3 Results

7.3.1 SCRS Group - There were no statistically significant differences in ATP content across time compared with the baseline (t=0) value. There were significant decreases in ADP and AMP content at 60-240 minutes and 120-240 minutes, respectively (Figure 7.1A).

7.3.2 SFRS Group - Both ATP and ADP significantly decreased across time. ATP content significantly decreased at 240 minutes and ADP was significantly

decreased at 60-240 minutes, compared with baseline values. There were no changes in AMP content across time (Figure 7.1B).

7.3.3 AA/D Group - Significant decreases in adenine nucleotides were observed in tissues exposed to 50 μM AA. Compared with baseline values, ATP and ADP were significantly decreased at 60-240 minutes and 120-240 minutes, respectively. There was an initial significant increase in AMP content at 60 and 120 minutes, which was followed by a significant decrease at 240 minutes (Figure 7.2 A). When compared to 60 minute values, 120, 180 and 240-minute ATP, ADP and AMP concentrations were significantly decreased.

7.3.4 AA/D-AA/R Group - Adenine nucleotide content followed a similar pattern as AA/D. However, no significant decrease in AMP was observed at 240 minutes (Figure 7.2 B). When compared with the 60 minute value, ATP concentrations at 120, 180 and 240 minutes were not significantly different. However, ADP and AMP followed a pattern similar to the AA/D group.

7.4 Discussion

In the presence of glucose and oxygen, equine colonic mucosal ATP content was stable in vitro for up to 4 hours. However, both ADP and AMP decreased. When glucose was not present, ATP content remained stable for only 3 hours. Addition of AA (50 μM) caused a time-dependent decrease in adenine nucleotide content. Removal of AA-containing Ringer's solution with subsequent solution replacement and addition of glucose prevented further depletion of ATP. However, unlike cell culture systems in which ATP is repleted upon removal of AA and addition of substrate (4), we did not observe ATP repletion in our whole tissue model.

Figure 7.1 (A and B) - Mean (±SEM) ATP, ADP and AMP content in equine colonic mucosal tissue. SCRS - substrate-containing (10 mM glucose) Ringer's solution; SFRS - substrate-free Ringer's solution. * denotes significant difference from time 0. $p \leq 0.05$ considered significant.

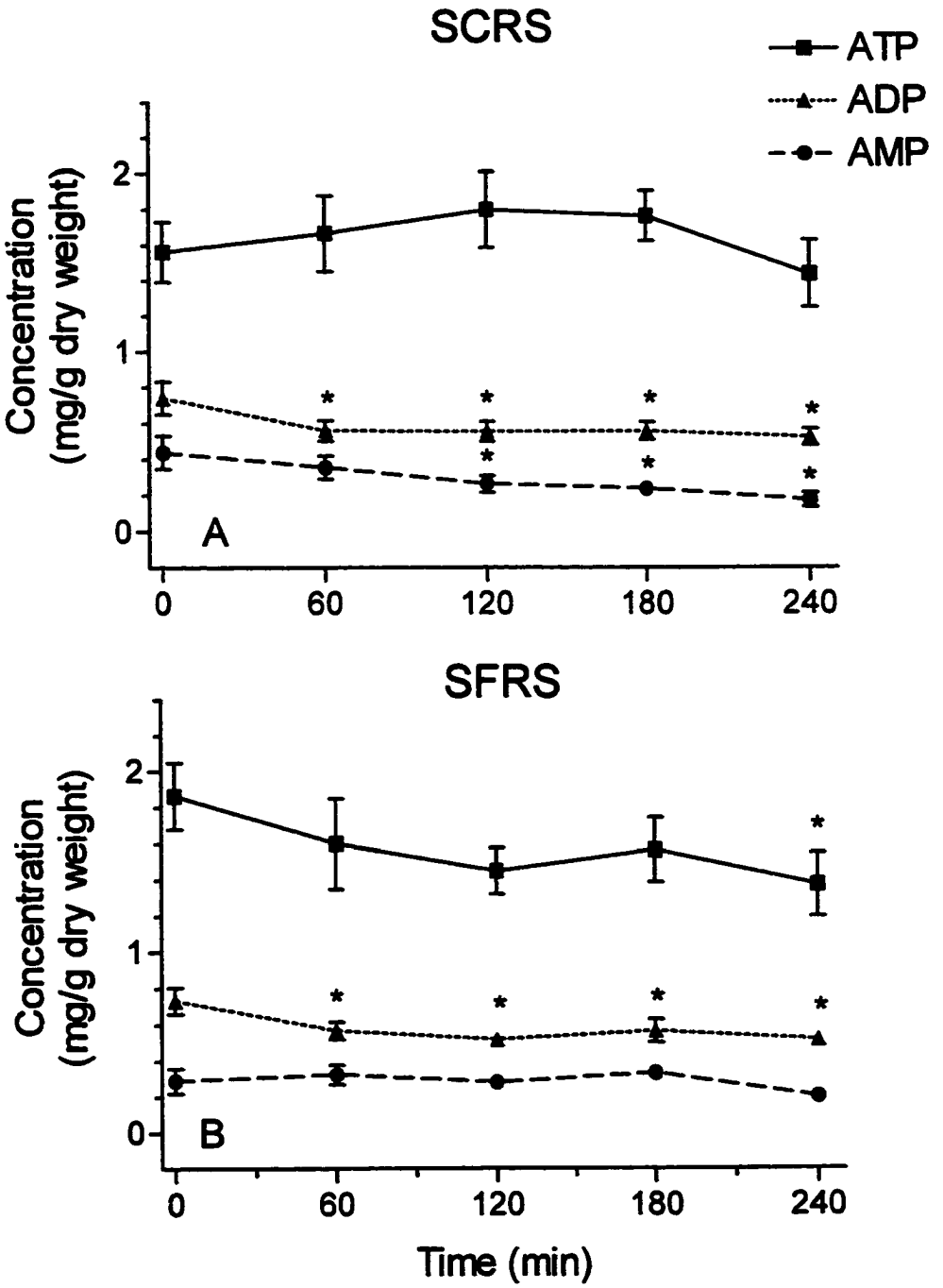
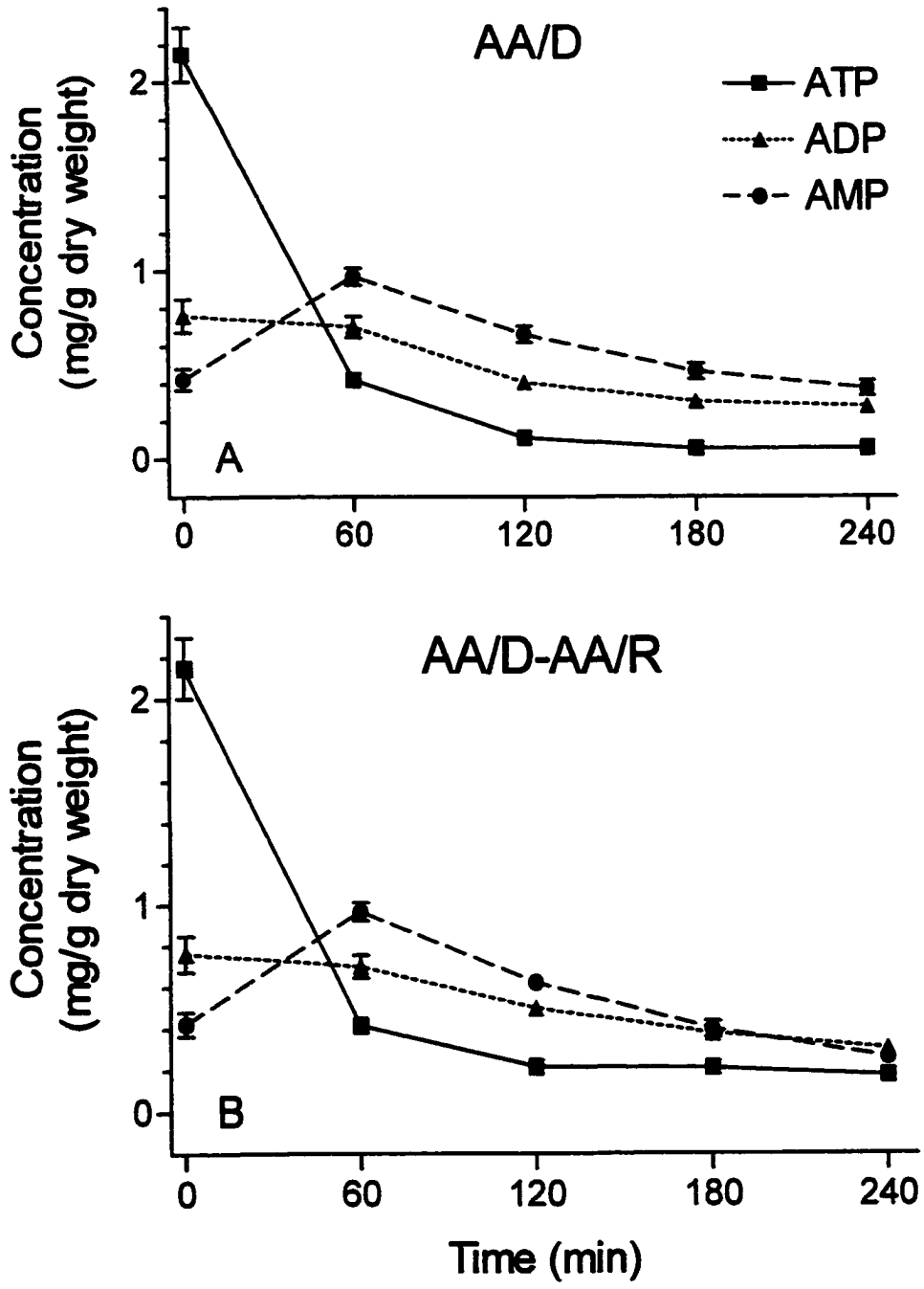


Figure 7.2 (A and B) - Mean (±SEM) ATP, ADP and AMP content in equine colonic mucosal tissue. AA/D - 50 μM antimycin A (AA) in SFRS; AA/D-AA/R - 50 μM AA in SFRS for 60 minutes followed by removal of AA and replacement with SCRS.
AA/D group: Significantly different from time 0 - ATP (60-240 min); ADP (120-240 min); AMP (60, 120, and 240 min). Significantly different from time 60 - ATP, ADP, and AMP (120-240 min).
AA/D-AA/R group: Significantly different from time 0 - ATP (60-240 min); ADP (120-240); AMP (60 and 120 min). Significantly different from time 60 - ADP and AMP (120-240 min).
$p \leq 0.05$ considered significant.



The intestinal epithelial tight junction restricts the paracellular permeation of ions and nonelectrolyte substances (13). In one study using chemical hypoxia to deplete ATP in cultured human intestinal epithelial cells, transepithelial resistance to passive ion flow rapidly decreased, and the effect was reversible if control conditions were restored within 1 hour (14). The decrease in resistance was demonstrated to be due to an increase in paracellular, not transcellular, permeability (14). Clinically, ATP depletion secondary to gastrointestinal tract ischemia may increase paracellular permeability, leading to passage of bacteria and endotoxin into the systemic circulation. Development of an in vitro reversible model of ATP depletion in equine gastrointestinal mucosal tissue would enable investigators to characterize the role of ATP and other adenine nucleotides in the regulation of paracellular permeability. Additionally, potential therapies aimed toward increasing cellular ATP content to maintain TJ, as well as cellular, integrity can be evaluated.

The reason(s) for the lack of reversibility of ATP depletion in our whole tissue model, compared with cell culture systems, is unknown, but several possibilities exist. First, the binding of AA to the tissue may be irreversible. Second, the cells may not be able to take up and utilize the glucose that was added to the in vitro system following removal of AA. Third, additional nutrients which were not present in the electrolyte solution that we used, but are present in cell culture medium, may be required for cells to synthesize ATP. Finally, tissues contain large quantities of ectonucleotidases, which degrade adenine nucleotides (15). The mucosal cells may have regenerated ATP, but the ATP may have escaped into the extracellular environment, subjecting it to

enzymatic degradation to a form (ie. adenosine, inosine, hypoxanthine) which was not measured in this study.

In conclusion, equine colonic mucosal ATP content appears to be stable in an in vitro system for a minimum of 3 hours in the presence or absence of substrate. Addition of an electron transport inhibitor markedly decreased ATP content. However, the depletion of ATP in our whole tissue model was not reversible. Additional studies using other mitochondrial (and glycolytic) inhibitors and different substrates need to be performed to determine whether a whole tissue in vitro model of reversible ATP depletion can be developed.

7.5 Product Information

[a]Beuthanasia, Schering-Plough Animal Health Corp., Union, NJ.

[b] G8270, Sigma-Aldrich Inc., St. Louis, Mo.

[c]A8674, Sigma-Aldrich Inc., St. Louis, Mo.

[d]FreeZone 4.5 liter freeze dry system, Model 77510, Labconco Corporation, Kansas City, MO

[E]Ultra-turrax T25, Tekmar Corp., Cincinnati, Ohio.

[f]Nalgene 4-mm syringe filters #176-0045, Nalge Company, Rochester, NY.

[g]Absorbosphere C18 5U column, Direct-connect prefilter kit #28689, All-guard cartridge system #96041, Alltech Associates, Deerfield, IL.

[h]HP 1090 liquid chromatograph, Agilent Technologies, Wilmington, DE.

[i]SAS version 8.0, SAS Institute, Cary, NC.

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SUMMARY

Intravenous infusion of a combination of ATP and $MgCl_2$ to clinically normal, conscious, adult horses significantly increased CO (0.35 to 0.8 mg of ATP/kg of body weight/min), decreased MAP and SR_L (0.35 to 1.0 mg/kg/min), and caused mild pulmonary hypertension (0.15, 0.3, and 0.4 to 0.9 mg/kg/min) without a significant change in PR_L. Magnitude of the hemodynamic alterations was dependent on the rate of infusion. The maximal safe infusion rate was 0.3 mg/kgt/min. All horses became lethargic, and their appetities diminished at infusion rates ≥ 0.4 mg/kg/min. Five of 6 horses exhibited signs of mild abdominal discomfort at infusion rates > 0.7 mg/kg/min. Flank sweating was observed in all horses at rates ≥ 0.45 mg/kg/min. Urine volume and specific gravity and hematologic, biochemical, and arterial blood gas alterations were detected during and after the infusion. The various hemodynamic and metabolic alterations were without appreciable detrimental effects at rates of infusion ranging from 0.05 to 1.0 mg/kg/min.

In clinically normal, anesthetized, adult horses administered IV ATP-$MgCl_2$ (0.1 to 1.0 mg/kg/min), systemic and colonic vascular resistance decreased in a rate-dependent manner. The decrease observed was principally via vasodilatation. A rate of 0.3 mg/kg/min caused a significant decrease in systemic and colonic arterial pressure and colonic vascular resistance without a signficant corresponding decrease in colonic arterial blood flow. No consistent signficant alterations in plasma colonic NO concentrations were observed, despite profound vasodilatation of the colonic arterial vasculature. The results suggest that IV infusion of ATP-$MgCl_2$ combination to horses

with gastrointestinal tract ischemia may be beneficial in maintaining colonic perfusion if driving pressure is maintained above a critical level.

In clinically normal, conscious, adult horses, IVATP-$MgCl_2$ (dose - 100 μmole ATP/kg and 100 μmole $MgCl_2$/kg; rate - 0.3 mg/kg/min) after low-dose (35 ng/kg) endotoxin administration failed to attenuate the clinical, hemodynamic, metabolic and hematologic alterations that occur secondary to endotoxin exposure. The combination of endotoxin and ATP-$MgCl_2$ appeared to potentiate the pulmonary hypertension, leukopenia, and neutropenia that occurred when endotoxin was given alone. Finally, endotoxin and ATP-$MgCl_2$ combination led to thrombocytopenia.

In isolated colonic arterial and venous rings collected from clinically normal horses, exogenous ATP caused a concentration-dependent relaxation response. At higher concentrations of ATP (10^{-6} tp 10^{-3} M), the vascular response to ATP was biphasic, with an initial, rapid and transient contraction followed by relaxation. Significant relaxation was detected at 10^{-4} M and 10^{-3} M. Removal of endothelium attenutated but did not eliminate the relaxation response in both arteries and veins. The mean maximum percentage attenuation of the relaxation response for endo - arteries and veins was 45.17% and 30.31%, respectively. The contribution of nitric oxide to the relaxation response was minimal and appeared to be more appreciable in veins. The L-NAME study paralleled our in vivo colon study, where no significant increases in either plasma colonic arterial or venous nitric oxide concentrations were detected despite the presence of profound vasodilatation of the colonic vasculature. The mechanism of action of the endothelium-dependent component of the relaxation response to ATP may involve PGI_2 or EDHF. However, nitric oxide cannot be definitely ruled-out. The ATP

used was not stable and, therefore, was subject to degradation to other adenine nucleotides (ie. adenosine), which would activate different mechanisms to cause endothelium-dependent vasodilatation.

We validated an established method for adenine nucleotide separation for use on equine colonic mucosal tissue utilizing HPLC. The limit of quantitation was 0.05 μg/ml. The method provided reproducible results when the tissues were ground into a homogenous samples prior to extraction. No significant decreases in adenine nucleotides were observed for at least 54-days when samples were lyophilized within 24 hours of collection and stored in a dessicator at -70 °C.

Finally, we evaluated the stability of adenine nucleotides in equine colonic mucosal tissue in vitro and a potential reversible model of adenine nucleotide depletion in a whole tissue model. In the presence of glucose and oxygen, ATP was stable for ≤ 4 hrs. However, both ADP and AMP decreased. When glucose was absent, ATP remained stable for ≤ 3 hrs. Addition of 50 μM antimycin A (electron transport inhibitor) caused a time-dependent decrease in adenine nucleotide content. Removal of antimycin A and addition of substrate prevented further depletion of ATP. However, adenine nucleotide repletion was not observed in our whole tissue model.

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APPENDIX: LETTERS OF PERMISSION

LOUISIANA STATE UNIVERSITY
AND AGRICULTURAL AND MECHANICAL COLLEGE
Veterinary Clinical Sciences • School of Veterinary Medicine

January 12, 2001

American Journal of Veterinary Research
The American Veterinary Medical Association
1931 N. Meacham Road, Suite 100
Schaumburg, IL 60173-4360

Re: Tetens J, Bueno AC, Cornick-Seahorn JL, Hosgood G, Eades SC, Moore RM. Hemodynamic and metabolic alterations associated with intravenous infusion of a combination of adenosine triphosphate and magnesium chloride in conscious horses. *AJVR*,1999;60-1140-1147.

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Sincerely,

Joanne Tetens

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January 12, 2001

American Journal of Veterinary Research
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Sincerely,

Joanne Tetens

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Diane A. Fagen 2/22/2001

Diane A Fagen **Date**
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VITA

Joanne Tetens was born on November 30, 1964, in Huntington, New York, United States. She grew up in New York and graduated from Northport High School in 1982. Joanne attended the State University of New York at Farmingdale, earning an associate's degree in veterinary science in 1984. In 1985, she transferred to Cornell University in Ithaca, New York, where she received a bachelor's degree in animal science in 1987. In 1988, Joanne was accepted into the veterinary medical curriculum at Oklahoma State University in Stillwater, Oklahoma. She graduated with honors in 1992 with the degree of Doctor of Veterinary Medicine.

From July 1992 to June 1993, Dr. Tetens did an internship in large animal surgery at the University of Pennsylvania's New Bolton Center, located in Kennett Square, Pennsylvania. Following the internship, she completed and equine surgical residency and master's degree program in Veterinary Clinical Sciences at Michigan State University, East Lansing, Michigan, in 1996. Dr. Tetens worked in equine private practice in 1996-97.

In October 1997, Dr. Tetens joined the faculty in the Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana, as a Clinical Instructor in equine surgery. In July 1998, she entered graduate school at Louisiana State University while continuing to work as a Clinical Instructor in emergency equine medicine and surgery. In 1999, Dr. Tetens became a Diplomate of the American College of Veterinary Surgeons. Dr. Tetens will be awarded the degree of Doctor of Philosophy in Veterinary Medical Sciences in May 2001.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Joanne Tetens

Major Field: Veterinary Medical Sciences

Title of Dissertation: Systemic and Colonic Hemodynamic and Vasomotor Responses to Adenosine Triphosphate in Horses

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

March 20, 2001